CLINICAL AND MOLECULAR GENETIC STUDIES IN FAMILIAL ADENOMATOUS POLYPOSIS AND COLORECTAL CARCINOMA

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THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF MEDICINE.

UNIVERSITY OF EDINBURGH.
1991

DEDICATION

I dedicate this thesis with all my love to my wife, Margot, and to my daughters, Kathryn and Lisa.

DECLARATION

I declare that this thesis was composed entirely by myself and that the work presented is my own unless otherwise stated. I have included some data generated by co-workers in my research group which strengthen my own findings presented in the sections on allele loss studies in sporadic adenomas and flow cytometric analysis carried out on my own collection of tumours.

Malcolm Graham Dunlop
December 1990

Presentation of this work was awarded the Edinburgh School of Surgery Chiene Medal, 1988 and the Surgical Research Society of Great Britain and Ireland Patey Prize, 1989.

The following publications derived from the work presented in this thesis are in press or in final preparation:

1. M.G. Dunlop, C.M. Steel, A.H. Wyllie, C.C. Bird and H.J. Evans. Linkage analysis in Familial Adenomatous Polyposis: Order of C11P11 (D5S71) and pi 227 (D5S37) loci at the *APC* gene. **Genomics 1989; 5: 350-353.**
2. P.G. Ashton-Rickardt, M.G. Dunlop, Y. Nakamura, R.G. Morris, C.A. Purdie, C.M. Steel, H.J. Evans, C.C. Bird and A.H. Wyllie. High frequency of *APC* loss in sporadic colorectal carcinomas due to breaks clustered in 5q21-22. **Oncogene 1989; 4:** 1169-1174.
3. M.G.Dunlop and C.M. Steel. Colon cancer: molecular analysis marches on. Editorial. **Lancet 1989; 1: 1236-1238.**
4. M.G. Dunlop. Inheritance of colorectal cancer susceptibility. **Br. J. Surg. 1990; 77: 245.**
5. N.D. Hastie, M. Dempster, M.G. Dunlop, A.M. Thompson, D.K. Green and R.C. Allshire. Telomere reduction in association with colorectal carcinoma and ageing in humans. **Nature 1990; 346: 866-868.**

6. Status of the *APC* gene in familial and sporadic colorectal tumours as determined by closely flanking markers. **Hereditary Colorectal Cancer. p453-456. Utsunomiya J and Lynch HT (Eds.). Springer-Verlag Tokyo 1990.**

7. M.G. Dunlop, A.H. Wyllie, Y Nakamura, C.M. Steel, H.J. Evans and C.C. Bird. Genetic linkage map of 6 polymorphic DNA markers around the gene for Familial Adenomatous Polyposis on chromosome 5. **Am. J. Hum. Genet. 1990; 47: 982-987.**

8. M.G. Dunlop. Allele losses and onco-suppressor genes. *In press.* **J. Pathol.**

9. MG. Dunlop, CM Steel and AH Wyllie. Clinical application of linked DNA markers for presymptomatic diagnosis of Familial Adenomatous Polyposis. *In press.* **Lancet.**

10. MG. Dunlop, PG. Ashton-Rickardt, AH Wyllie, Y.Nakamura, CA. Purdie, RG Morris, J Piris, CM Steel, HJ Evans and CC Bird. The involvement of the Familial Adenomatous Polyposis locus in the colorectal adenoma-carcinoma sequence. In final preparation for submission to **Br. J. Cancer.**

Abstracts

11. Familial Adenomatous Polyposis (FAP): Gene order at the FAP gene locus with evidence to support the hypothesis that the germline mutation is sufficient for the development of colonic adenomata. **Br.J.Surg. 1989; 76: 625.**

12. Gene probe analysis of the locus at 5q21 involved in Familial Adenomatous Polyposis (FAP) **Proceedings of 158th Meeting Pathological Society of Great Britain and Ireland, London, Jan. 1989.**

13. Loss of the 5q21 Familial Polyposis allele in sporadic colorectal cancer. **Proceedings of 158th Meeting Pathological Society of Great Britain and Ireland, London, Jan. 1989.**

14. Fine linkage map around the Adenomatous Polyposis (*APC*) gene. **Cytogenet. Cell Genet. 1989; 5: 1049.**

15. A chromosome 5 deletion map in non-familial colorectal carcinomas. **Br. J. Surg. 1989; 76: 1346.**

16. Human colorectal cancer, but not breast cancer, shows a high frequency of chromosomal telomere shortening. **Br.J.Surg. 1990; 77: A694.**

17. Presymptomatic diagnosis of familial adenomatous polyposis by DNA probe analysis. **Br.J.Surg. 1990; 77: A698.**

18. Chromosome 5 allele losses in familial and sporadic colorectal adenomas and carcinomas. **Br.J.Surg. 1990; 77: A705.**

19. Molecular genetics and colorectal cancer susceptibility. **Proceedings of King's Fund Forum Consensus Symposium: Cancer of the colon and the rectum, London 18-20th June, 1990**

ABSTRACT

The work presented in this thesis employs clinical and molecular genetic techniques to study genetic aberrations involving the gene for Familial Adenomatous Polyposis (FAP) in colorectal carcinogenesis and in the clinical syndrome of FAP.

Epidemiological data are reviewed and demonstrate that colorectal cancer is a major public health problem in westernised societies, with little improvement in mortality for 50 years. Putative aetiological factors are briefly reviewed and the evidence for the adenoma-carcinoma sequence in colorectal carcinogenesis is presented. The heritable syndrome of FAP is described in detail and is proposed as an *in vivo* model of sporadic colorectal carcinogenesis. The gene for FAP (*APC*) was previously regionally mapped to chromosome 5 and has been implicated as a tumour suppressor gene, involved in both the familial and sporadic forms of colorectal cancer. A review of epidemiological, statistical, cytogenetic, molecular genetic and somatic cell hybridisation evidence supports the existence of tumour suppressor genes and data specifically relating to *APC* are examined. Knowledge of *APC* involvement in sporadic and familial colorectal neoplasms prior to the commencement of this work is discussed.

Fifteen probands affected by FAP were identified and eleven of these kindreds were exhaustively ascertained and the clinical FAP phenotype of all affected family members fully evaluated. Nine families served as the resource for genetic linkage analysis. Blood leukocyte and archival paraffin-embedded pathology tissue DNA from family members were analysed by Southern blot and DNA hybridisation techniques. 155 family members (90 meioses) were genotyped for DNA probes exhibiting restriction fragment length polymorphisms (RFLP's). A genetic linkage map of 6 RFLP probes was constructed around the *APC* gene by genetic linkage analysis and by identification of crucial recombinants. These are the first data which have allowed mapping of all of these markers and support a locus order of centromere-pi227-C11P11-ECB27-L5.62-*APC*-EF5.44-YN5.48-telomere.

This work was the first to apply these markers to the clinical care of FAP families. Preclinical diagnosis of FAP was carried out in 41 at-risk family members (aged 0-39 years). The clinical value and validity of DNA probe derived risk estimations were demonstrated. Four at-risk family members who had been discharged from clinical screening were shown to be affected by DNA analysis and cancer prevented by prophylactic colectomy. The calculated residual risk for the majority of those apparently unaffected was sufficiently low as to allow a reduction in the frequency of colonic screening. Recommendations are made for an integrated approach to screening for FAP.

The linkage map suggests that the probe EF5.44 is very close to *APC* and so analysis of large DNA fragments at the EF5.44 locus in 2 sporadic cases of FAP using pulse field gel electrophoresis was carried out. However, no rearrangements were detected up to 800kb from the EF5.44 locus in the cases examined and so final mapping of the *APC* gene using PFGE in this study was not possible.

The high resolution map of 6 DNA probes around *APC* gene generated by genetic linkage analysis was used to examine the frequency, nature and extent of losses of genetic material from that chromosome in FAP and non-FAP colorectal neoplasms. A further 7 polymorphic DNA markers spanning chromosome 5 were also examined. Loss of constitutional heterozygosity in tumour DNA was assessed at all 13 loci using blood leukocyte and normal colonic mucosa DNA as controls. A deletion map was then constructed for each tumour which exhibited any chromosome 5 allele losses. The composite deletion map generated from allele loss data in carcinomas allows independent ordering of many of the markers and the locus order derived from linkage analysis is supported. There were no chromosome 5 allele losses in 28 informative FAP adenomas. Twenty of these adenomas were informative at markers flanking the *APC* gene and spanning a distance of as little as 2 megabases. This stringent assessment of *APC* gene status in FAP adenomas suggests that the inherited *APC* mutation allows adenomatous change in the colonic epithelium of FAP patients without the requirement for a 'second hit'.

Fifty one sporadic colorectal carcinomas were analysed. *APC*-related allele loss was demonstrated in 67% of carcinomas informative at any locus and in 56% of tumours where markers closely flanking the *APC* gene were both informative. The nature of the defects in chromosome 5 were small interstitial deletions (64%), mitotic recombination events (33%) and whole chromosome loss (3%). There were no particular associations between chromosome 5 allele loss and clinico-pathological features or ploidy level by flow cytometry, suggesting the early involvement of *APC* in colorectal carcinogenesis.

Twenty sporadic adenomas were collected and an overall loss of heterozygosity of 17% at any chromosome 5 locus was demonstrated. There was a highly significant difference in the allele loss frequency between sporadic adenomas and FAP adenomas. There was also a statistically significant difference in allele loss frequency detected at any informative chromosome 5 locus between sporadic adenomas and carcinomas. The data suggest that inactivation of the *APC* gene is permissive of early tumour development and appears to be involved in the selection of cells within an already adenomatous colonic epithelium for progression to malignancy.

The role of *APC* defects in the FAP syndrome and in colorectal carcinogenesis has been investigated and a number of novel discoveries presented. The gene has been mapped to a small region of chromosome 5q and this region analysed in benign and malignant colorectal neoplasms. The demonstration of the consistent and specific deletion of *APC* emphasise its importance in colorectal carcinogenesis.

ACKNOWLEDGEMENTS

This project has involved input from a great many professional colleagues and from many patients who were more than willing to help with research.

I would like to thank Professor HJ Evans and Dr. CM Steel of the MRC Human Genetics Unit, and Professor CC Bird and Dr. AH Wyllie of Edinburgh University Pathology Department for their help and support which allowed me to carry out this work based at the MRC Human Genetics Unit. The project received financial support from a Medical Research Council Training Fellowship.

The experimental work presented in this thesis was very much part of a research group effort which comprised the MRC Unit and Edinburgh University Pathology Department. This combination proved highly fruitful and provided an excellent milieu for research. I have learned a great deal from the many disciplines involved.

I would like to thank Susan Collyer and Rhona de May for their patience and persistence in searching the public records for FAP family members. Dr G Sclare proved an invaluable assistance in tracing FAP family members through the pathology records at Bangour General Hospital and I gratefully acknowledge his help and advice. I am most grateful to all of the family doctors and hospital clinicians who allowed me access to their patients and in general for their active support of the project. Professor CI Phillips and Mr. S Gairns carried out a great many retinal examinations and fundal photography and I am most grateful to them for their invaluable assistance. I also thank Dr.K Wood for his expert assessment of the orthopantomograms.

I spent a great deal of time in the Pathology Department at the Western General Hospital and I would like to thank all of the pathologists there, and in particular Dr. M. MacIntyre, for their help in providing me with representative tumour samples and for increasing their own workload to ensure histological assessment of the tumour tissue adjacent. I am indebted to Dr. Juan Piris for his expertise in the histopathological assessment of the adenomas harvested from FAP patients. I would also like to thank all of the pathologists from various centres who provided archival paraffin block material for deceased FAP family members. I am indebted to Colin Purdie for his help and expertise in carrying out flow cytometry on all of my series of colorectal neoplasms.

Any success I have had in this project has been partly due to the enormous amount of help and practical advice I have had from many colleagues in the MRC Human Genetics Unit who taught me what little molecular genetics I now know. I would particularly like to acknowledge James Mackay and Paddy Elder who

introduced me to basic techniques, and David Porteous, Julia Dorin, Wendy Bickmore and John Maule who patiently taught me some of the more involved techniques such as PFGE and helped sort me out when I had experimental difficulties or problems with interpretation of data. I am also indebted to Eric Thompson for his expertise and experience in chromosome analysis and I am most grateful to Liz Harvey and Michael Steel for their invaluable work in transforming and maintaining the lymphoblastoid cell lines. I thank all of those workers who donated DNA probes used in this project and particularly acknowledge the collaboration with Yusuke Nakamura. His staggering ability to clone enormous numbers of polymorphic DNA markers allowed this project to produce such detailed linkage and deletion maps. I am also most grateful to Susan Holloway who patiently taught me to understand and use the LINKAGE programs.

I would like to thank Norman Davidson, Douglas Stuart and Sandy Bruce of the photography department at the MRC Human Genetics Unit for their excellent photography and illustration work.

I acknowledge Dr. Andrew Wyllie in his official capacity as my supervisor for the purposes of this thesis but I thank both Andrew and Michael Steel most of all for their friendship, enthusiasm, advice and support throughout this project. I am also indebted to them for their helpful comments during the preparation of this manuscript. Without them, I would never have been involved in the field and the success of this project is largely due to them.

ABBREVIATIONS

APC	The human gene mapping term for the defective gene causing the FAP syndrome.
ATP	Deoxyadenosine triphosphate
bp	Base pairs (of DNA)
BSA	Bovine serum albumin
BudR	Bromodeoxyuridine
CHRPE	Congenital hypertrophy of the retinal pigment epithelium
CTP	Deoxycytidine triphosphate
dH_2O	Distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetic acid
FAP	Familial Adenomatous Polyposis
FCS	Fetal calf serum
GTP	Deoxyguanidine triphosphate
kb	kilo base pairs (of DNA)
PBS	Phosphate buffered saline
PFGE	Pulse field gel electrophoresis
PHA	Phytohaemagglutinin
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SSC	Salt and sodium citrate buffer
TAE	Tris Acetate EDTA buffer
TBE	Tris Borate EDTA buffer
TCA	Trichloroacetic acid
TE	Tris EDTA
TTP	Deoxythymidine triphosphate

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CHAPTER 1

INTRODUCTION

This chapter reviews the importance of colorectal cancer as a major public health problem in the developed world and discusses putative aetiological factors related to diet. Evidence is presented which demonstrates that a heritable predisposition to colorectal cancer exists and there appears to be a genetic-environmental interaction. A brief review is given of current research into possible methods of improving the prognosis of colorectal cancer.

The evidence for the adenoma-carcinoma sequence and the importance of the adenoma as a premalignant lesion is reviewed. Understanding the fundamental molecular changes in genetic material that result in adenomatous and then carcinomatous change in the colonic epithelium might lead to novel treatment approaches and perhaps even to identification of individuals at high risk of developing colorectal malignancy. Evidence is presented that the syndrome of Familial Adenomatous Polyposis (FAP) is a genetic condition reflecting the events involved in non-polyposis colorectal carcinogenesis. Hence FAP might be considered a model of the neoplastic process in the colorectum. The clinical syndrome of Familial Adenomatous Polyposis is described in detail with particular emphasis on stigmata which can be used to identify gene carriers before the onset of polyps. Evidence is presented that the gene for Familial Adenomatous Polyposis (*APC*) is a tumour suppressor gene and that the genes for retinoblastoma and Wilms' tumour represent paradigms for such genes. The statistical, epidemiological and experimental evidence for such tumour suppressor genes is reviewed. Specific genetic aberrations reported in FAP and in colorectal cancer are discussed with particular reference to possible tumour suppressor gene involvement. In the light of that knowledge, the aims of this project are presented and the experimental approach described.

1.1 Clinical aspects of colorectal carcinoma and Familial Adenomatous Polyposis.

1.1.1 The incidence and geographic distribution of colorectal cancer.

Carcinoma of the colon and rectum is predominantly a disease of westernised societies (Burkitt 1971, Haenszel 1971). It is the second most common cause of death due to cancer in the United Kingdom, second only to lung cancer. 25,000 new cases are diagnosed each year and around 19,000 patients die from the disease annually. Scotland is the region with the highest incidence of colorectal cancer within the UK (Kemp 1985) with a crude rate of 52/100,000 (male and

female) and an age standardised incidence in males of 34.2/100,000 (cf. Birmingham (33.0), Oxford (30.7), South Thames (28.0)). The incidence rate in Scottish females is also high (27.5) when compared to other regions within the UK. Within Scotland there are striking geographical aggregations of cases and these differences in incidence rates are highly statistically significant (Kemp 1985). There is a very high rate for males in the Tweedale district (80.4/100,000 crude rate, 55.3 standardised rate) and for males and females in north-east Scotland (around 85/100,000 crude rate, 50 standardised rate) with a four-fold difference between the highest and lowest incidences within Scotland. These discrepancies remain unexplained but may suggest a local high exposure to an environmental risk factor or a high prevalence of a cancer predisposing gene, perhaps through relative in-breeding within the local population.

The incidence rates for colorectal cancer in the United Kingdom as a whole, and Scotland in particular, are high in comparison to other countries (Waterhouse 1982). There is generally a tenfold increased incidence in North Western Europe and North America when compared with developing countries such as sub-Saharan Africa, India and many regions of South America (Burkitt 1971, Doll 1980, Haenszel 1971, Waterhouse 1982). The age-standardised incidence rate for males in Connecticut, USA is 20 times greater than that for males in Senegal (Waterhouse 1982). No other malignancy so closely parallels the affluence of a society in its incidence around the world.

In westernised countries, colorectal cancer is a disease primarily affecting those in the 7th and 8th decades of life (Cancer Registration Statistics, Scotland, Goligher 1984, Kemp 1985). Less than 5% of cases arise in patients under 35 years of age (Goligher 1984) and when the disease occurs in such young individuals, there is frequently a family history of colorectal cancer.

The Scottish incidence rate is rising (Cancer Registry Statistics, Scotland 1971-1980). Surgical treatment has achieved only a modest improvement in mortality from the disease since the classical publications on tumour staging and outcome (Dukes 1929-30, Dukes 1931, Dukes 1940, Lockhart-Mummery 1926) The minimal improvements in outlook for these patients (Bailar 1986, Gill 1978, Goligher 1984, Giles 1982) are probably due mainly to a reduction in operation-related mortality, with improved anaesthetics and post-operative support. Therefore, the number of patients dying of colorectal cancer has remained substantially unchanged in 55 years (1930-1985) (Registar General (Scotland) 1985). Clearly, colorectal cancer is a significant health problem in westernised countries, both in terms of its incidence and also due to the overall poor prognosis with less

than one third of patients alive 5 years after presentation (Falterman 1974, Gill 1978, Goligher 1984, Jass 1987).

Since colorectal cancer is a major health problem which has been little influenced by modern medical care, it would seem logical to gain greater understanding of the aetiological factors involved in order to develop novel treatment approaches. What is already known about the cause of colorectal cancer?

1.1.2 Aetiological factors in colorectal cancer.

The high incidence of colorectal cancer in developed countries has been attributed to environmental factors present in the western diet. The evidence for this comes from geographic variations in the incidence of the disease (Burkitt 1971, Doll 1980, Haenszel 1971, Waterhouse 1982) and from studies which show that migrant populations assume the incidence of the indigenous population within 2-3 generations (Haenszel 1971). The dietary factors implicated as carcinogens have been identified in the main by experimental colorectal carcinogenesis and are not always supported by case-control or cohort studies (Bruce 1987). Burkitt (1971) suggested that a high fibre intake reduces the intestinal transit time and dilutes potential faecal carcinogens. Hence the exposure of the colonic epithelium to carcinogens might be reduced. However there is virtually no evidence to support this in man or in laboratory animals (Stubbs 1983). A low fibre and high fat content in the diet appears to result in high faecal pH which has been shown to enhance bile acid toxicity. Cellular proliferation also appears to be reduced when the faeces are acidified (Bruce 1987). However there have been no direct tests of the effect of faecal pH manipulation on colonic epithelial proliferation. Only one study has assessed the influence of faecal acidification by ingestion of vitamins C and E on the development of colorectal adenomas or carcinomas and this revealed no effect (McKeown-Eyssen 1988). However, the time period of the study was relatively short and the authors themselves suggest a further larger and more prolonged study. High dietary intake of animal fat has been suggested as an important aetiological factor and its mode of action may be via an increase in faecal bile acids with gut bacterial conversion to carcinogenic compounds related to methylcholanthrene (Stubbs 1983). Bile acids themselves have been shown to have a toxic effect on colonic epithelium and high fat intake mobilises bile acids. However there are no clear differences apparent in dietary fat consumption between colon cancer cases and a control population in case-control studies (Bruce 1987, Stubbs 1983). In addition there is evidence to suggest that individuals with low serum cholesterol are actually at higher risk of colorectal cancer (Doll 1981), although the association of plasma cholesterol concentration and colorectal cancer mortality appears to be 'U'-

shaped with an increased rate of colorectal cancer deaths at cholesterol concentrations above and below the mean population level (Isles 1989).

It has been suggested that the bio-availability of bile acids is important. Calcium has been proposed as an agent which might reduce the availability of bile acids. Dietary calcium supplements have been shown to reduce colonic epithelial proliferative indices to normal levels in patients at high risk of colorectal cancer, such as those with multiple polyps and affected members of colon cancer families (Lipkin 1985, Rozen 1989, Wilson 1990). However international case-control studies have so far failed to show a clinical effect (Bruce 1987).

A summary of the state of understanding of aetiological factors in colorectal cancer is difficult since the field is full of contradictory and negative evidence. For the present, there remains little doubt that diet is of great importance in the high incidence of colorectal cancer in the western world but the exact factor or factors involved still require to be elucidated. As a result, it is not yet possible to recommend rational dietary intervention. Until further epidemiological, case-control and interventional studies identify the agents responsible, we must look to other methods of reducing colorectal cancer mortality.

1.1.3 Towards reduction in mortality from colorectal cancer.

There is a need for novel approaches to reducing the mortality from colorectal cancer. Three broad strategies are possible: alteration in treatment modalities, identification of the disease in a pre-malignant phase or at an early, treatable stage and dietary intervention with the aim of preventing neoplastic change in the first place.

Optimising current surgical management

Aggressive surgical treatment regimes have been advocated. Some studies have reported a beneficial effect of radical surgery involving extended lymphadenectomy (Koyama 1984) and radical excision of the mesorectum (Heald 1986). These studies have suggested that survival can be improved and/or local recurrence reduced, but there are no properly controlled randomised trials and so at present the results of ever more radical surgery cannot be assessed objectively. In addition, significant morbidity and mortality may well be associated with such extensive surgery. At a more practical level, there are substantial differences in the outcome for patients with colorectal cancer dependent on the surgeon who carried out the operation (Phillips 1986) and on certain crucial aspects of technique (Quirke 1986). This suggests that improvements in survival are possible by identifying the key operative techniques employed by those surgeons producing the best results and applying these universally.

The generally poor outlook following surgery for colorectal cancer has prompted many authors to propose adjuvant therapy as a necessary part of treatment (Gill 1978). Two adjuvant treatment modalities have been evaluated but the results have been of mixed success and the message concerning use of these modalities in routine clinical practice remains somewhat unclear.

Adjuvant therapy

Radiotherapy has been shown to be of no value in cancer of the colon (Lancet editorial 1985). However, this is not the case for rectal cancer, where benefit has been demonstrated by randomised controlled trials (reviewed by Buyse 1988, Cummings 1989, Duncan 1985, Gaze 1988). Several of these trials have shown a statistically significant reduction in the incidence of pelvic recurrence (Gerard 1985, MRC 3 Trial, Stockholm Rectal Cancer Group 1987). However, local recurrence is by no means eradicated by pelvic irradiation and so adjuvant radiotherapy is by no means routine clinical practice. It has also proven difficult to establish whether adjuvant radiotherapy confers survival benefit in rectal cancer. The results of all trials of radiotherapy published up to 1986 in English (3062 patients) were reviewed by Buyse (Buyse 1988) and failed to show a statistically significant improvement in survival. The most recent results from the MRC trial of adjuvant radiotherapy in rectal cancer have shown a significant reduction of pelvic recurrence from 22% in controls to 8% in those in the radiotherapy arm ($p<0.001$) but the reduction in mortality from 21% to 13% just fails to achieve statistical significance ($p=0.06$) (Prof. WJ MacKillop, personal comm.) The trend towards a reduction in mortality in several studies has lead to the suggestion that very large trials are now required (Buyse 1988). It is important to note that the proven benefit in reducing local recurrence and potential benefit of reduction in mortality are gained at a cost since there is morbidity associated with pelvic irradiation.

Chemotherapy has also been assessed as a means of improving outcome (Buyse 1988, Hunt 1989, Wooley 1988). Only regimes containing 5-fluorouracil have shown any benefit. A met-analysis of all trials of adjuvant chemotherapeutic regimes containing 5-FU up to 1986 (4700 patients) demonstrated that there is only a 2.3-5.7% improvement in 5-year survival and this does not achieve statistical significance (Buyse 1988). Very recently, a trial of adjuvant 5-fluorouracil combined with levamisole has shown significant benefit in survival for patients in the treatment arm (Moertel 1990). This trial demonstrated a reduction in death rate of 33% for patients with Dukes stage C disease ($p=0.006$). Clearly this is a step forwards but the results of this trial must be interpreted with caution until verified by independent studies. It is also important to note that the survival rate of the

treated patients was still only 60% and so this drug combination clearly cannot be considered as the ultimate in adjuvant treatments.

Although there is a rationale for treatment which might supplement surgical excision of colorectal cancer, there is no incontrovertible evidence demonstrating benefit and so, for the present, routine use of such adjuvant therapy must be left to the individual clinician involved in the patients care. If the MRC Trial of adjuvant radiotherapy for rectal carcinoma does subsequently show a significant benefit and the combination of 5-FU and levamisole in Dukes stage C disease is confirmed by independent studies then this may well change treatment policy in the near future. Even if proven beneficial, these treatments will remain as adjuvants modalities with surgery as the mainstay of treatment for the foreseeable future.

Screening for malignant and premalignant colorectal lesions.

Surgical treatment of early colorectal cancers is generally highly successful, the majority of patients surviving 5 years (Dukes 1929-30, Dukes 1932, Fielding 1986, Jass 1987). However, such early cancers are relatively rare and the majority of tumours have already metastasised at the time of clinical presentation (Dukes 1929-30, Falterman 1974, Gill 1978, Goligher 1984, Jass 1987). Therefore, the potential benefit of surgical treatment is limited by the natural history of the disease. It is reasonable to assume that detection of the disease at an early stage might overcome this problem and hence the rationale of screening for colorectal cancer has been born (Hardcastle 1989b).

Detection and removal of premalignant polyps and excision of cancer before it has spread intuitively seems to offer the best hope of a reduction in mortality from colorectal cancer. However, there are no prospective controlled data to support this. The study reported by Gilbertson (Gilbertson 1974) does appear to show benefit but the data are uncontrolled. Therefore it must be assumed that early detection of cancers will indeed follow the pattern of those tumours arising in patients reported in retrospective studies.

Screening for colorectal cancer can be approached by screening the whole population or by targeting high risk groups for more intensive screening procedures. Such cancer screening programs are subject to a number of potential sources of bias: *lead-time bias* arises where a tumour is simply diagnosed earlier but without affecting its natural history and so the disease is sub-clinical for less time and clinical for longer; *length bias* occurs where less aggressive tumours which grow slowly are identified by screening rather than aggressive tumours which become clinically apparent very quickly and so those patients with screen-detected cancers appear to do well when compared to the whole patient group with colorectal cancer. However, such patients would have done better in any case since the natural history

for their disease was of a less aggressive time course. A further potential bias is of *patient selection* where those likely to respond to an invitation for screening may be more health conscious and perhaps have a diet which is fundamentally less likely to favour the development, or even the progression, of colorectal cancer. Therefore, the screened population would have a reduced incidence of colorectal cancer simply because they responded to the screening invitation in the first place. Such sources of bias can only be overcome by assessing the effect of screening for colorectal cancer in a randomised controlled trial over a long time course.

Four randomised controlled trials of population screening with faecal occult blood testing are currently under way (Gilbertson 1980, Hardcastle 1989a, Kewenter 1988, Kronberg 1987). The largest of these is the Nottingham trial with 107,349 subjects randomised to date with a follow-up period of 6 months to 5 years (average 3 years) (Hardcastle 1989a). Preliminary data from this study are encouraging with more favourable prognostic indicators consistently shown in screen-detected cancers versus the control group. The incidence of cancer in the control group was 0.72/1000 patient years whereas an initial screen revealed 8 cancers per 1000 subjects screened. Hardcastle calculates that the sensitivity of occult blood screening is around 55%. There were also large numbers of adenomas detected and treated in the study population which may well lead to a long term reduction in the incidence of colorectal cancer in this group. The proportion of Dukes stage A cancers detected in the screened population (53%) was significantly higher than for those arising in the control group (11%). Screen detected cancers also showed a trend towards more favourable degree of tumour differentiation and fixity. The proportion of severely dysplastic adenomas was also lower in the screened versus the control groups. However, despite all of these encouraging signs, it is too early to tell whether there is actually a survival benefit for the screened versus the control population and so population screening by faecal occult blood testing should not be recommended until the trials of Hardcastle and others are completed.

The practicalities of implementing a population screening service with the knock-on effects on colonoscopic and radiological services are daunting. Another promising way ahead may be to identify and offer screening to individuals who are at very high risk of colorectal cancer as the return of cases identified would be high and the subjects positively motivated in the knowledge of their high risk. Such a program has recently been carried out with a high level of compliance. Five patients with cancer and 62 with polyps were identified of 382 high risk patients who were colonoscoped (Houlston 1990). Empiric risks had been derived from previous data on family history of colorectal cancer and the risk for each patient was then

calculated on the basis of their individual family history. However, this study has all the problems of being wholly uncontrolled.

The method of screening of high risk groups merits comment. The St. Mark's group found that faecal occult blood testing was of no value in detecting cancers in their study population and so they strongly recommend colonoscopy as the screening procedure of choice. Hardcastle's group have also assessed the value of faecal occult blood screening of high risk groups with a strong family history of colorectal cancer and found occult blood testing did not identify colorectal neoplasms with any greater frequency than in a control screened population not selected on the basis of family history (Armitage 1986). It would therefore appear that the screening tool best suited to individuals at high risk due to possible inheritance of a colorectal cancer is colonoscopy. However, two previously reported controlled studies of screening for colorectal cancer using colonoscopy in high risk sub-groups suggest little, if any, benefit for the screened population (Grossman 1988, Grossman 1989). These studies were somewhat unsatisfactory due to poor control data and the yields of neoplasms were assessed only at a single screen. It was not established whether there was any long term benefit to screened individuals. There is clearly a need for a controlled long-term trial of intensive screening for colorectal cancer in subjects with a strong family history of large bowel malignancy.

Dietary manipulation

The third possible method of reduction of colorectal cancer mortality is to reduce the overall incidence of the disease by dietary manipulation. This is an attractive proposition but the aetiological factors involved have not been fully established and this is discussed in section 1.1.2. Nationwide abandonment of current dietary habits for an African-style diet is clearly impossible. However, interventional studies of dietary manipulation based on current knowledge are under way. Only one randomised clinical trial has been published to date (McKeown-Eyssen 1988). It assesses the recurrence of colonic adenomas following polypectomy in patients with and without dietary supplementation of vitamins C and E . Such anti-oxidants were thought to reduce the level of the mutagenic substances in the stools by alteration of faecal pH. However, no significant difference was noted in recurrence rate between the two groups. Further studies have examined the effect of added dietary calcium on colorectal epithelial cell proliferation in patients at high risk of colorectal cancer (Lipkin 1985, Rozen 1989, Wilson 1990). These studies demonstrated a reduction in cellular proliferation to levels seen in individuals at low risk. However there are no published clinical data which demonstrate benefit derived from dietary calcium supplementation. The way

ahead must now be to identify which dietary constituents have the potential to reduce the carcinogenic effect of the western diet and to test their putative beneficial effect in large interventional controlled trials.

There may not be the political will or the economic and practical capability for a national alteration in dietary habits but if it were possible to identify a high risk sub-population, it might well be possible to alter the diet of such a selected group of patients. Criteria identifying populations of individuals at high risk of colorectal cancer are already well established. Family history indicates a strong genetic input and this is discussed below in section 1.1.7. A pathological marker of high risk of large bowel cancer is the adenomatous polyp and the theme of this work is based around the adenoma-carcinoma sequence with particular reference to the syndrome of Familial Adenomatous Polyposis in which that progression can be studied.

1.1.4 The adenoma-carcinoma sequence in colorectal carcinogenesis.

The evidence for the adenoma-carcinoma sequence in the development of colorectal malignancy is substantial, if not overwhelming. Practical and ethical considerations mean that adenomas are not left *in situ* and simply observed repeatedly until malignancy supervenes. Therefore, the evidence indicating that the adenoma as the benign precursor of colorectal carcinoma usually relies on indirect evidence. Notwithstanding this, the evidence from observational data is so convincing as to be beyond all reasonable doubt and this is reviewed here.

Demographic variations in incidence of colorectal carcinoma closely parallel the demographic incidence of adenomas. The prevalence of colorectal adenomas in an autopsy study in Liverpool, with its high rate of large bowel cancer, (Williams 1982) was very much higher than the prevalence in Nigeria, where colorectal cancer is uncommon (Williams 1975). There is also a parallel increase in the prevalence of colorectal adenomas and carcinomas when a population from a low incidence area migrates to an area with a high incidence. The prevalence of adenomas in native Japanese over the age of 50 is 27% (Sato 1976) whereas Hawaiian-Japanese have a prevalence of 63% in the same age-group (Stemmerman 1973). Large bowel adenomas are very uncommon in indigenous African populations such as Nigerians (Williams 1975) and the South African Bantu (Bremner 1970). However the prevalence of adenomas in American negroes in New Orleans is actually higher than in the white population in the same city (Correa 1977).

The topographical distribution of carcinomas and the larger adenomas along the length of the colorectum also exhibits a strong degree of concordance. Approximately 66-75% of colorectal cancers arise in the rectum and sigmoid colon (Falterman 1974, Goligher 1984) and there is also a predilection of large adenomas

for these regions (Williams 1982). In a series of 1187 adenomas, 47% occurred in the sigmoid colon with the proportion of adenomas decreasing proximally to the right colon (8.2%) (Morson 1983). In this study and that of Williams (1982), the proportion of adenomas greater than 1cm was higher in the sigmoid than elsewhere in the colon although this was not the case for adenomas less than 5mm in diameter which were distributed evenly throughout the colon and rectum.

Benign, adenomatous tissue adjacent to malignant tumour in resected colorectal cancers is well described. In one study, 14.2% of malignant tumours were partly composed of benign tissue (Muto 1975). The proportion of malignant tumours which contain a benign element has been shown to be related to the extent of spread of the carcinoma through the bowel wall. In early carcinomas, limited to the submucosa, the frequency of detecting a benign component is 57%, with spread limited to the bowel wall, it is 18% and where there is transmural involvement there is benign tissue detectable in only 8% of malignancies (Morson 1966). In addition, *in-situ* carcinoma has never been demonstrated without adjacent benign tissue (Lane 1976). This is strong evidence to suggest that the natural history of the malignant tumour involves the replacement of a benign precursor by invasion and/or destruction (Lane 1976).

Approximately 30% of colorectal cancer resection specimens contain adenomatous polyps, again suggesting a association. This is further supported by the fact that the life risk of developing a second (metachronous) cancer in the residual bowel is around 5% overall but 10% in those cases where polyps were present in the resected specimen (Heald 1975, Morson 1984, Muto 1975). The risk also increases with increasing numbers of polyps to 70% if 6 or more polyps were present (Muto 1975). Similarly, the incidence of associated polyps in colons resected for synchronous cancers was 50% (Heald 1975). Such associations of benign and malignant colorectal neoplasms strongly suggest that the benign lesion is the precursor of the malignant one. Another explanation of such associations is that the carcinogenic effluent in the colon may simply favour the development of neoplasms, both benign and malignant. However, the weight of other evidence would argue against this hypothesis.

The age distribution for the diagnosis of adenomas and carcinomas also suggest that the benign lesion predates the malignancy. In one study, the average age of onset of a single adenoma (58.1 years) preceded the average age of onset for a single carcinomas (62.1 years) by around 4 years (Muto 1975). However this may underestimate the true difference as adenomas are frequently symptomless for many years.

The autosomal dominant syndrome of Familial Adenomatous Polyposis, described in detail in section 1.1.5, may represent a model of sporadic colorectal carcinogenesis and this is discussed in section 1.1.6. The hundreds or thousands of adenomatous polyps which characterise this disorder render the affected individual at such high risk of cancer that prophylactic colectomy is routinely advised. The cancer risk associated with this syndrome again supports the notion that the polyp is the precursor of the cancer in the colorectum.

Further supporting evidence for the adenoma-carcinoma sequence is the demonstration that sigmoidoscopic screening and prophylactic removal of rectosigmoid adenomas is paralleled by an apparent reduction in the incidence of carcinomas in the rectosigmoid in the screened population (Gilbertson 1974). However, this study did not include a control arm and requires confirmation as discussed in section 1.1.3. Anecdotal clinical evidence also exists of a carcinoma developing at the site of a documented adenoma which had not been excised.

There is also direct molecular genetic evidence to support the adenoma-carcinoma sequence and the parallel increase in malignant potential with increasing size and villous component of the adenoma. *Ras* gene mutations, mainly consisting Ki***ras*** mutations can be detected in 40-50% of colorectal cancers (Bos 1987, Delattre 1989, Forrester 1987, Vogelstein 1988). It has been shown that in small adenomas with low grade dysplasia, *ras* mutations can be demonstrated in only 13% of tumours. However, in the larger, more cytologically aggressive lesions, the frequency of *ras* mutations is the same as for that in carcinomas (Vogelstein). A further crucial piece of evidence comes from the analysis of Ki***ras*** mutations in carcinomas with residual adenomatous tissue, where mutations can be demonstrated in both the adenoma and carcinomatous tissue (Vogelstein). It is also important to consider that all of these studies show that such events occur sometimes and clearly does not happen every time. Hence not all individual adenomas are premalignant.

All of the above evidence strongly supports the concept of the adenoma-carcinoma sequence and much of the rationale for the work presented in this thesis is based on this premise. The well-defined genetic disorder of Familial Adenomatous Polyposis alluded to above allows study of the adenoma-carcinoma sequence at a molecular genetic, as well as a pathological level.

1.1.5 Clinical aspects of Familial Adenomatous Polyposis.

Familial Adenomatous Polyposis is an autosomal dominant disorder which is characterised by the development of multiple colorectal and other gastrointestinal neoplastic polyps with almost certain progression to colorectal cancer without

appropriate treatment. The syndrome is not solely an intestinal one, affecting optic retina, thyroid, bone, skin and connective tissue. This section describes and discusses clinical aspects of FAP with particular regard to the early diagnosis by screening for colonic and extra-colonic manifestations of the disorder such that prophylactic surgery can be undertaken to minimise the risk of cancer.

Historical and pathological review

The first case of Familial Adenomatous Polyposis (FAP) appears to have been reported in 1881 (Sklifasowski 1881) and the realisation that the disorder might be inherited came in 1882 when the disease was reported in a brother and sister (Cripps 1882). Malignant progression was first described by Smith (Smith 1887) and now the risk of colorectal cancer is recognised to be so high in these patients (approaching 100%), that prophylactic colectomy is required (Bussey 1975, Morson 1974, Murday 1989, Muto 1975).

The disease is characterised by the presence of more than 100 adenomatous polyps of the rectum and colon with the average number being around 1000, although the polyps can number up to 5000 (Bussey 1975). Carcinoma will almost inevitably supervene unless prophylactic colectomy is performed. The pathology of the adenomatous lesions has been well documented at the macroscopic, microscopic (Bussey 1975, Morson 1983) and ultrastructural level (Mughai 1978). The adenomas and the carcinomas which supervene show similar pathological features to those seen in non-FAP cases. The various histological types of adenoma (tubular, tubulo-villous and villous) are found in similar proportions in familial and non-familial cases (Morson 1983) and the carcinomas are comparable as regards tumour site, stage, degree of differentiation, and outcome (Utsunomiya 1978). The only difference appears to be that of number of polyps. The very high risk of development of colorectal cancer in FAP with a much lower risk in non-FAP cases might at first seem to be one major difference. However, when the large number of adenomas present in the colon of a patient with FAP is considered along with the fact that there is usually only one or perhaps two carcinomas arise in a complicated case, it is clear that the risk of malignant conversion of any one FAP adenoma is actually quite low. Hence the frequency of malignant conversion in FAP and non-FAP adenomas come more into line.

Epidemiological, genetic aspects and screening

The burden of FAP in the general population is difficult to estimate due to problems of disease registration but the incidence is probably around 1/10,000 (Bulow 1987, Bussey 1975, Reed 1955). The disease appears to be distributed around the world with comparable frequency (Bulow 1987, Hill 1982, Reed 1955, Utsunomiya 1978).

Familial Adenomatous Polyposis is inherited as an autosomal dominant disorder (Bussey 1975, Reed 1955). A substantial proportion of affected individuals have no affected relatives and the majority of these are sporadic cases due to new mutations within the gene for FAP. The overall proportion of such new mutations appears to be around 40% (Bulow 1987, Bussey 1975, Utsonomiya 1978). This high mutation rate suggests that the gene involved may be quite large, offering a large target for mutagenic events. The offspring of apparent new mutations also have 50% risk of inheriting the mutant gene. Gene penetrance is age dependent with the peak age incidence for presentation of polyps depending on whether the population studied was screened for the disease or not (Bulow 1987, Bussey 1975, Murday 1989). The correct assessment of age-dependent penetrance is important since risk estimations are partly derived from such calculations. The age of onset of polyps is frequently biased upwards as what is measured is the age when polyps first present clinically. The correct assessment of age-dependent penetrance is only possible by calculations based on a screened population as described by Murday (Murday 1989). Penetrance approaches 100% at 60 years of age (Bulow 1987, Murday 1989). The peak age of detection of the onset of adenomas for screened and non screened individuals is 23.7 and 27 years respectively. The same figures for the onset of carcinomas is 33 and 39.2 years respectively (Bussey 1975). It is clear that there is a 'window' when the disease can be diagnosed in a premalignant stage and so all offspring of affected individuals must be screened for the disease as their risk is 50% of having inherited the gene defect.

The value of screening is demonstrated by comparing the incidence of carcinoma in non-screened individuals which approaches 100% (Bussey 1975) and the incidence in call-up patients of 3-9% (Bulow 1987, Bussey 1975). Screening for the disease in at-risk individuals involves clinical examination of the rectum and sigmoid colon by rigid or flexible sigmoidoscopy. More involved tests such as colonoscopy or barium contrast studies are unnecessary as a routine since rectal polyps inevitably occur even in cases where colonic polyps are the initial presentation (Bulow 1987). Clinical screening of at-risk family members should involve yearly examinations from puberty to the age of 35 and then 3-5 yearly until the age of 60 years (Bulow 1987).

Prophylaxis in gene carriers

The rationale for early detection of Familial Adenomatous Polyposis is that prophylactic colectomy can be offered to affected individuals at a stage before malignancy supervenes. Surgery involves removal of all, or the majority, of the premalignant colorectal mucosa. This is achieved by proctocolectomy, subtotal colectomy and ileorectal anastomosis, or total colectomy and ileo-anal anastomosis

with an ileal pouch reservoir serving as a neo-rectum. Proctocolectomy was considered to be the operation of choice in many centres until recently as it offers complete removal of the premalignant colorectal epithelium but it has the disadvantage of leaving the patient with a permanent ileostomy. A further disadvantage of proctocolectomy is that at-risk relatives may be adversely influenced by the prospect of a stoma such that as to avoid surveillance and hence expose themselves to risk of malignancy. The procedure is still commonly carried out on FAP patients, especially when rectal cancer has already developed. Proctocolectomy was carried out 48% of FAP cases in one series (Bulow 1987) but in only 14% in the St. Mark's series (Bussey 1985) which reflects a special interest in colectomy with ileorectal anastomosis at that centre. Colectomy with ileorectal anastomosis is now considered the method of choice in controlling the onset of malignancy in FAP patients. It allows normal defaecation although the stool may be a little more loose and frequent and also avoids post-operative problems with bladder control, potency or fertility which can occur rarely with any pelvic excision. There are disadvantages to this procedure and these are due to the persistence of rectal mucosa which allows further adenomas to develop. However, this can be controlled by life-long surveillance and cautery of rectal adenomas (Bussey 1975). There remains a risk of development of malignancy in the retained rectum and this has been estimated at 13% at 10 years (Bulow 1984) in one series and 13% after 25 years with a cumulative risk of dying of rectal cancer of only 4% at 30 years (Bussey 1985). Colectomy with ileorectal anastomosis should also be considered in patients with rectal adenomas at the time of diagnosis as there are well documented cases of polyp regression following the procedure (Bussey 1975, Hubbard 1957, Williams 1966). This phenomenon has been more rigourously assessed and the phenomenon of polyp regression is now well established (Nicholls 1988). Polyp regression does not reduce the need for careful surveillance of the retained rectum but does reduce the need for repeated fulguration. There is little to be lost by performing colectomy and ileorectal anastomosis since recurrence of rectal polyps can still be treated by proctocolectomy or the more recently described operation of total colectomy and mucosal proctectomy with ileal pouch-anal anastomosis (Parks 1980). The functional results from this operation are good in experienced hands (Nicholls 1985), but it should probably only be carried out in specialist centres. The patients are able to defaecate normally although the frequency is usually increased to greater than 4 movements daily (Nicholls 1985). Post operative problems are common and for the present it is reasonable to consider this as a second line treatment of Familial Adenomatous Polyposis if recurrent polyps are a problem after colectomy with ileorectal anastomosis.

Extra-colonic features of FAP

Familial Adenomatous Polyposis is a multisystem disorder which indicates the constitutional nature of the genetic defect. The various clinical features which make up the syndrome are not only related to the colon and serve to emphasise this point.

Multiple polyposis is well described in the upper gastrointestinal tract. Gastric polyps have been detected in 70% of FAP patients in some studies (Bulow 1987, Fausa 1989, Utsonomiya 1978) and in 55% of patients in one prospective screening study (Spigelman 1989). However, it has been shown that only a minority (8%) of gastric polyps are adenomas (Spigelman 1989), while the majority show cystic enlargement of the fundic glands characteristic of fundic gland polyposis, which does not amount to adenomatous change. Duodenal polyposis is also very common and was demonstrated in 93% of patients in one study (Fausa 1989). More conservative estimations of the incidence of duodenal adenomas suggest that the incidence is around 50-70% (Bulow 1985, Burt 1984). However, in Spigelman's prospective study of 102 FAP patients (Spigelman 1989), while macroscopic duodenal polyps could be discerned in 86% of cases, on random biopsy histological changes of dysplasia or hyperplasia were demonstrated in 98% of patients. The periampullary region was particularly prone to polyp formation and Spigelman attributes these findings to the effects of bile on the upper gastrointestinal mucosa. Duodenal and or peri-ampullary carcinoma is also part of the FAP syndrome (Jagelman 1988, Jones 1980, Pauli 1977). It has been estimated that there is up to a 200-fold increased risk of this tumour in FAP patients (Pauli 1977) and the overall life risk of periampullary cancer for patients cured of their colonic disease is around 7% (Bulow 1985, Bussey 1972). One large study from the Leeds Castle Polyposis Group (Jagelman 1988) consisting of 1255 patients showed a prevalence of invasive upper gastrointestinal cancer of 4.5%, with duodenal and periampullary carcinoma making up 68% of all cases. Because of the risk of upper GI malignancy, it has been advocated that FAP patients should be followed up indefinitely by upper GI endoscopy at 5-yearly intervals. Unfortunately it is not clear if screening and intervention will have any effect on mortality from upper GI malignancy in FAP and it is somewhat alarming to consider the prospect of a prophylactic pancreatico-duodenectomy! However, it is pertinent to consider that after sub-total colectomy and ileorectal anastomosis, FAP patients are more likely to die of upper GI cancer as of carcinoma of the retained rectum.

The presence of small bowel adenomas has also been reported in FAP (Bulow 1987, Utsonomiya 1978) but the incidence of progression to malignancy appears to

be very low since very few cases of small intestinal carcinoma in FAP patients have been reported to date (Jagelman 1988, Ross 1974).

The association of bone tumours and Familial Adenomatous Polyposis was first noted by Gardner (Gardner 1951, Gardner 1952) and these lesions have subsequently been shown to be osteomas (Ooya 1976). This association, combined with epidermoid cysts (*vide infra*), was thought to be a distinct clinical syndrome and was given the eponym Gardner's Syndrome. However, it has been shown that cranio-facial osteomas are very commonly associated with adenomatous polyposis. In controlled studies using orthopantomography, osteomas have been demonstrated in 81%-93% of cases (Utsunomiya 1975, Ushio 1976) and it is now routine practice in some centres to carry out bone surveys in all FAP patients (Utsunomiya 1978). The median number of mandibular osteomas in FAP patients is 2, with a range 1-6 (Bulow 1984b) The appearance of osteomas tends to precede the onset of polyps by 18-20years (Duncan 1968). In at-risk individuals, the appearance of osteomas can be taken to indicate that the patient is indeed affected, even if polyps have yet to become apparent. In individuals who have multiple osteomas but no family history of FAP, serious consideration should be given to the need for screening of the colon as the patient may be a sporadic case of FAP. The high frequency of cranio-facial osteomas in FAP has led some authors to suggest that ortho-pantomography of the mandible could be used as a screening tool for at-risk individuals (Bulow 1987). However, the incidence of osteomas in the general population is between 4% (Bulow 1985, Sondergaard 1985) and 16% (Utsunomiya 1975) and so, at worst, screening for FAP in at-risk family members by such a modality would have a sensitivity of 76% (Bulow 1985, Jarvinen 1982); a specificity of 82% (Utsunomiya 1975); a predictive value of a positive test of 81% and a predictive value of a negative test of 77%. In addition, no studies have addressed the possibility that the appearance of these lesions is age-dependent. Hence, screening for FAP by searching for osteomas cannot be used in isolation but can be used as an aid to conventional screening, as suggested by some authors (Utsunomiya 1978).

There are no recorded cases of malignancy developing in the osteoma of a patient with FAP. However, these lesions can cause local pain and discomfort. Relief of symptoms can be achieved by surgical excision of troublesome tumours, but in general the tumours are best left alone.

The association of desmoid tumours with Familial Adenomatous Polyposis was first described by Smith (Smith 1958). These lesions are classified with other fibromatous conditions such as Dupuytren's contracture and keloid scar but their exact nature is not clearly understood (Anderson 1978). When not associated with FAP, desmoids tend to arise in the rectus abdominis muscle of multiparous women

and compress and destroy muscle tissue without metastasising (Anderson 1978, McAdam 1970). The incidence of desmoid tumours in FAP patients is around 4% (Bulow 1986, Bussey 1975, Smith 1958) and 89% occur in women (Bulow 1986). These capricious lesions occur intra-abdominally in 51% of cases (Bulow 1986) and can obstruct the ureters and other intra-abdominal viscera (McAdam 1970). They can be sensitive to hormonal manipulation but in general no treatment has any lasting benefit and the patient progressively deteriorates as the tumour continues to compress, obstruct and destroy intra-abdominal organs. The lesions can cause a great deal of pain and discomfort which can be difficult to control. Surgical excision has little to offer as frequently the tumour progresses even more quickly after surgical manipulation. Desmoid disease is one of the great disappointments in the treatment of FAP patients since patients may undergo successful restorative proctocolectomy only to succumb to the effects of a 'benign' lesion which causes great distress and frequently results in the patients demise. This problem may become more common with the increasing success rate of screening programs and timely surgery for true gastrointestinal malignancy which allows more and more FAP patients to avoid early death due to cancer.

Multiple epidermoid cysts are also part of the FAP phenotype originally thought to indicate the presence of Gardner's syndrome but, as in the case of osteomas, when an active search is made, these lesions are common in all forms of Familial Adenomatous Polyposis. The term 'sebaceous cyst' has been used to describe these lesions but since this is a generic term including epidermoid and pilar cyst as well as steatocystoma multiplex (pilo-matrixoma), the lesions are best referred to as epidermoid cysts which are the only type associated with FAP (Leppard 1975). They occur in 53% of cases of FAP (Leppard 1974) and in one study 33% had one or more lesions removed in childhood (Leppard 1975). The cysts tend to occur on the face and scalp in FAP patients but more frequently on the back in non-FAP cases (Leppard 1974). The mean number of lesions has been reported to be 4 but with up to 20 in one individual (Leppard 1974). The appearance of an epidermoid cyst on the face or scalp of a prepubesent child should be regarded with some interest as these lesions are very uncommon in this age group when not associated with Familial Adenomatous Polyposis.

A pigmented lesion of the optic retina known as Congenital Hypertrophy of the Retinal Pigment Epithelium (CHRPE) has recently been described in association with Familial Adenomatous Polyposis. CHRPE lesions consist of multiple, isolated, flat, well-demarcated, hyperpigmented areas which frequently affect both eyes. The hyperpigmented areas are usually surrounded by a hypo-pigmented halo and patchy depigmentation may occur within a hyperpigmented lesion. Histologically, the

lesions exhibit hypertrophy of the pigment epithelial cells with increase in the size of the pigment granules within these cells and degeneration of the overlying photoreceptors also occurs (Purcell 1975). Congenital Hypertrophy of the Retinal Pigment Epithelium does not appear to cause symptoms or affect visual fields or acuity. The CHRPE lesions are best visualised by indirect ophthalmoscopy as they are frequently peripheral. The first report of an association between CHRPE and Familial Adenomatous Polyposis was published in 1982 (Blair 1982). Since the original description, there have been many reports which have further defined this association (Chapman 1989, Diaz-Llopis 1988, Iwama 1990, Lynch 1987, Polkinghorne 1990, Stein 1988, Traboulsi 1987). The importance of the CHRPE phenotype lies in the possibility that eye examination might allow presymptomatic diagnosis of the disease. One study (Chapman 1989) suggested that 3 or more pigmented lesions, particularly when occuring in both eyes, was both a sensitive (100%) and specific (100%) test of FAP status. The controls in this study were not unaffected family members, but a totally unrelated group and so the true specificity of the test could not be assessed. Other authors have reported the sensitivity of the presence of CHRPE indicating inheritance of FAP as 55% (Iwama 1990), 97% (Polkinghorne 1990), 100% (Stein 1988) and 78% (Traboulsi 1987). Clearly CHRPE is a useful tool for demonstrating the presence of FAP but it is of lesser value in excluding inheritance of the mutant gene. It is also important to note that CHRPE lesions also occur in normal controls (Stein 1988, Traboulsi 1987) but whereas some patients with FAP have less than 3 CHRPE lesions, no normal controls have been shown to have more than 3 in one study (Chapman 1989) or more than 4 in another (Traboulsi 1987). It is clear however that some FAP families do not have CHRPE lesions as part of the FAP phenotype (Traboulsi 1987, Polkinghorne 1990). The presence of multiple typical CHRPE lesions in an at-risk individual must be considered to indicate the presence of the disease. The absence of such lesions can only allow confident presymptomatic disease-state prediction where affected members of the same family have been shown to have CHRPE lesions as part of the expression of the disease. There is also evidence that the number of pigmented lesions correlates with the age of onset of polyps (Chapman 1989) and so individuals who have been screened negative by colonic examination and do not have CHRPE lesions can probably be cautiously reassured and subjected to less intensive screening protocols. However the confidence with which this advice can be given could be greatly increased by an independent genetic test of disease state and this is an important part of the experimental work for this thesis.

Thyroid carcinoma has also been described in association with Familial Adenomatous Polyposis (Alm 1973, Plail 1987) and it would appear that women

below the age of 35 years with FAP are at 160 times greater risk of thyroid carcinoma than a healthy female counterpart. The histology tends to be predominantly of papillary type. Careful palpation of the thyroid must now be included in the follow-up protocol for young females affected by FAP, with early institution of fine needle aspiration and ultrasound screening of any suspicious nodules .

FAP is due to a constitutional gene mutation

It is clear from the above description that the syndrome of Familial Adenomatous Polyposis is not simply a colorectal polyposis, but clinical and pathological changes can be detected in most body tissues. This serves to highlight the fact that the inherited mutation is a constitutional phenomenon. The involvement of multiple tissues in the FAP phenotype suggests that the gene involved may have pleiotropic effects involving endoderm, mesoderm, and ectoderm and alteration in its function results in the diversity of the clinical syndrome. An alternative explanation is that there is a whole group of genes located physically close to the gene for FAP and that alteration in the gene for FAP such as by deletion would also influence the surrounding genes. The argument against this second explanation is that the clinical presentation of the syndrome should breed true in any given family since each affected family member would inherit the same characteristic group of deleted genes and this is clearly not the case, as discussed above. It seems most likely that the gene for FAP is involved in biological activity in many different tissues. Identification of the gene for Familial Adenomatous Polyposis will be of great interest, not least because of the widespread effects of the causative mutation. There is also evidence to suggest that the gene for FAP may have a key role in sporadic colorectal carcinogenesis.

1.1.6 Familial Adenomatous Polyposis as a model of sporadic colorectal carcinogenesis.

Familial Adenomatous Polyposis has long been considered a model of sporadic colorectal cancer at a pathological level (Lane 1963). As discussed in Section 1.1.5, there are striking similarities between the stages of neoplastic growth in colonic epithelium of FAP and non-FAP cases using a variety of modalities of investigation. The changes are similar with regard to the development of uni-cryptal adenomas (Oohara 1980) to large polypoidal lesions (Morson 1983). The different histopathological types of adenoma are found in similar proportions in familial and non-familial cases (Morson 1983). The stage, degree of differentiation, and the clinical prognosis of carcinomas are also comparable (Utsunomiya 1978). The

anatomical distribution of polyps and of cancer developing in FAP patients tends to be in the rectum and sigmoid and closely parallels that seen in sporadic adenomas and carcinomas (Bussey 1975, Utsunomiya 1978). The progression of adenomas to carcinomas also holds for Familial Adenomatous Polyposis. In one large series, 36% of 199 colorectal cancers arising in patients with FAP were partially comprised of a benign element compared with 14.2% for non-FAP carcinomas (Bussey 1975). The reason for this high rate of benign tissue is probably related to the early presentation of patients with cancers complicating FAP due to symptoms from the burden of adenomas rather than due to symptoms from the carcinoma itself. However the progression to malignancy in FAP is entirely in keeping with the notion that FAP is a model of sporadic colorectal carcinogenesis.

At a clinical level there is also evidence to suggest that FAP reflects the changes seen in sporadic colorectal cancer. There is a 10 year difference in the screen-detected peak age of detection of polyps without a cancer and the screen-detected peak age of detection of polyps complicated by cancer for patients with FAP (Morson 1983). The difference of peak age distributions for the diagnosis of a single polyp or cancer in non-FAP cases is at least 4 years, which is remarkably concordant when it is considered that a single polyp will remain asymptomatic for very substantially longer than the large number of polyps in the colon and rectum of a patient with FAP.

It can be seen from the discussion above that the process of neoplastic change in the colorectum of FAP patients does appear closely to mirror that which leads to sporadic colorectal cancer, the outstanding difference being only in the number of adenomas. It is certainly reasonable to propose that Familial Adenomatous Polyposis can be considered a human model of colorectal cancer. Study of the genetic factors underlying FAP is therefore important for two distinct but inter-related reasons. At the level of patient care, it may be possible to identify gene carriers and appropriate screening and treatment instigated. Clinicians in the United Kingdom have a high level of awareness of the need for screening and prophylactic colectomy for affected individuals and so cases of FAP-related colorectal cancer make up only a very small proportion of the total caseload of large bowel cancer in this country (<0.2%). Should changes in the gene responsible for the syndrome of FAP prove to occur in non-FAP colorectal cancers, then this may suggest that genetic factors have a far more wide-reaching influence on the development of colorectal cancer. Study of the genetic aberration(s) responsible for FAP may therefore lead to more fundamental understanding of the molecular mechanisms of sporadic colorectal carcinogenesis.

1.1.7 Evidence for a genetic influence on the development of sporadic colorectal carcinoma.

The existence of the syndrome of FAP indicates the involvement of a heritable gene defect conferring susceptibility to colorectal cancer. There are also clinical clues to the presence of genetic disorders which may involve other genes in addition to that causing FAP. Alternatively, a proportion of these syndromes may result from a different class of mutation within the gene for FAP. This section discusses the involvement of the gene for FAP in genetic susceptibility to non-FAP colorectal cancer.

There is little doubt from the discussion in section 1.1.2 that dietary factors are implicated in the aetiology of large bowel malignancy. There is substantial evidence to suggest that genetic susceptibility to colorectal cancer interacts with environmental factors. There are well documented pedigrees exhibiting an obvious autosomal dominant form of inherited colorectal cancer and these have been described by Lynch (Lynch 1985, Lynch 1988). These dominant colon cancer syndromes make up at least 5% of the burden of colorectal cancer (Mecklin 1987). Other dominant colon cancer syndromes exist with specific associations such as Muir's syndrome (Anderson 1980, Muir 1967) where multiple skin tumours are also inherited.

Many studies have shown familial clustering of cases of colorectal cancer (Bonelli 1988, Burt 1985, Cannon-Albright 1988, Duncan 1982, Lovett 1976, Macklin 1960, Woolf 1958, Woolf 1955) which is not restricted to cases in which a dominant gene appears to be operant, resulting in 50% of the offspring of an affected individual inheriting the disease (Hereditary Non-polyposis Colorectal Cancer). Such clustering of cases cannot be accounted for by the simple hypothesis of common exposure to high levels of environmental carcinogens since the incidence in spouses of affected patients is that of the general population (Cannon-Albright 1988, Jensen 1980). Published data support the existence of a gene defect transmitted in a Mendelian dominant mode resulting in colorectal adenomas and carcinomas with a population frequency of 19% (Cannon-Albright 1988). Since inheritance of the mutant gene only confers susceptibility to colorectal neoplasia, not all individuals with the gene defect develop colorectal cancer.

It seems likely that there may be more than one colorectal cancer predisposing gene but there are already some pointers as to the likely phenotype of this putative cancer susceptibility gene defect. Skin tags have been reported to be more common in patients with colorectal adenomas than in the general population (Leavit 1983, Kune 1985) although there is some contradictory evidence (Gould 1988) Although it is possible that carcinogens which come into contact with the

skin may also be consumed orally, it seems more likely that such patients have inherited a defect in a gene with pleiotropic effects involving skin and colonic epithelium. Mandibular osteomas are common in patients with Familial Adenomatous Polyposis (FAP) and these lesions also occur with a higher frequency in patients with apparently non-familial colorectal cancer than in a control population (Sondergaard 1985). Mandibular osteomas are also more common in patients with colorectal adenomas although the association does not achieve statistical significance (Sondergaard 1986). In some kindreds with cancer family syndrome there is also an increased incidence of mandibular osteomas (Sondergaard 1985). This evidence suggests that one of the 'colorectal cancer genes' may actually be the gene for FAP but perhaps carrying a different class of mutation within it. There has been recent molecular genetic evidence to suggest that a class of mutations in the gene for FAP may confer an increased risk of colorectal cancer to a much greater extent than that limited to the classical syndrome of FAP. Recently, genetic linkage analysis has been carried out in one large kindred (Leppert 1990) and linkage has been established to a disease gene resulting in variable numbers of adenomatous colorectal polyps using chromosome 5q DNA markers known to be linked to the gene for FAP which is now known as *APC*. While some patients clearly had FAP, many of the affected family members had only a very few colorectal polyps and so the type of *APC* mutation is this family has more subtle effects than that of the usual class of *APC* mutation. It is conceivable that other *APC* mutations may have the effect of inducing the formation of even more limited numbers of polyps and could be responsible for at least a proportion of the genetic influence on the incidence of colorectal cancer discussed above.

Identification of a gene or genes which predispose to colorectal cancer would have resounding implications for the screening and early detection of colorectal neoplasms in addition to the development of novel treatment approaches. Isolation of the gene for FAP may therefore have far reaching implications outside that syndrome alone.

1.2 The gene for Familial Adenomatous Polyposis (*APC*): Localisation and possible tumour suppressor activity.

The potential involvement of the gene for FAP (*APC*) in the genesis of colorectal cancer in patients FAP and in those with sporadic bowel cancer has been discussed above. Further assessment of the involvement of that gene in colorectal carcinogenesis will require localisation and ultimately cloning, sequencing and full characterisation of the non-mutant gene function. Much of the experimental work

in this thesis involves localisation of the *APC* gene and its assessment in sporadic colorectal cancers. However, final assessment of *APC* function must wait until its full characterisation. This section discusses a putative role for the *APC* gene as a so-called tumour suppressor gene and the evidence to support such a role.

1.2.1 The importance of identification of the *APC* gene.

Identification of the gene for Familial Adenomatous Polyposis is of great importance due to its causal involvement in a heritable cancer syndrome, its probable involvement in the biology of colorectal cancer and its possible implication as a gene predisposing to non-FAP colorectal cancer.

Following identification of the *APC* gene, presymptomatic diagnosis of FAP would be possible by a simple blood test rather than the protracted endoscopic screening program which is currently required. This would avoid screening the 50% of at-risk individuals who are currently having unnecessary examinations and would also relieve some of the psychological morbidity of uncertainty of diagnosis in patients who are actually affected.

The involvement of the *APC* gene in the biology of colorectal cancer is also of great importance. As described in section 1.1.6, the inherited mutation of FAP appears to result in the formation of multiple adenomas which have the same topographic distribution, histo-pathological and ultra-structural features, and the same natural history as the sporadic counterpart. The presence of each adenoma confers on that individual the same risk of developing a carcinoma within that particular polyp as the risk associated with sporadic adenomas. It is simply the large number of adenomas occurring at an early age which results in the high frequency of carcinomatous change in the colorectum of patients with Familial Adenomatous Polyposis (Morson 1983) and in the differences in clinical presentation. Therefore it is reasonable to hypothesize that the gene for Familial Adenomatous Polyposis might also be involved in some aspect of sporadic colorectal carcinogenesis and that *APC* involvement might well be an early occurrence.

The involvement of a gene defect resulting in a heritable cancer syndrome and also in the sporadic counterpart of that tumour was formally proposed by Knudson (Knudson 1971) (see section 1.3.1). Although specifically referring to retinoblastoma, Knudson has also suggested that the gene for Familial Adenomatous Polyposis might be implicated in sporadic colorectal cancer (Knudson 1989). The model proposed by Knudson suggests that two mutations are required to inactivate the target gene, one for each gene copy. Thus, although transmission of the disease shows a classical autosomal dominant pattern, these genes are

recessive at a cellular level. Supporting evidence for the existence of such tumour suppressor genes is reviewed in sections 1.3 and 1.4.

Another aspect of the *APC* gene is its possible implication as a colorectal cancer predisposition gene. The existence of a class of inherited *APC* mutations might well explain the prevalence of mandibular osteomas in apparently sporadic colorectal cancer and also might account for the familial aggregation of some cases of large bowel malignancy (see section 1.1.7).

1.2.2 Attempts to localise *APC* by cytogenetic analysis.

Demonstration of a cytogenetic abnormality in FAP in itself might define a marker of the disease for presymptomatic diagnosis. Disease state prediction using cytogenetic analysis is already in widespread clinical use for disorders such as Down's Syndrome where prenatal diagnosis is also possible. Perhaps more important in an autosomal dominant disorder involving a single gene defect such as FAP, identification of a cytogenetic lesion would provide a signpost to the chromosomal region harbouring the gene. This would allow detailed analysis at a sub-microscopic level using the techniques of modern molecular biology.

In 1982, a consistent deletion of chromosome 2 was reported in all of 17 karyotyped patients with FAP (Gardner 1982) but independent investigators were unable to confirm this cytogenetic aberration using high resolution chromosome banding techniques (Kasukawa 1983). The original authors also failed to repeat their own findings and ascribed the apparent aberration to artefact (Fineman 1984). Widespread chromosomal instability of cultured cells from FAP patients is well described (Danes 1978, Delhanty 1983) and so it is not surprising that such artefact was identified since it is well known to cytogeneticists that incorrect preparation of metaphase spreads in normal patients without chromosomal instability can give apparently consistent site-specific chromosomal lesions due entirely to artefact. An intensive study of 5 FAP patients using high resolution chromosome banding failed to show any karyotypic abnormality (Nielsen 1985).

A single case report of an interstitial deletion of 5q in a patient with multiple congenital abnormalities was the first clue to the whereabouts of the gene for Familial Adenomatous Polyposis (Herrera 1986). The deletion appeared to involve the region 5q13-q15 or 5q15-q22. Clearly this was an extremely fortuitous finding given the apparent rarity of such deletions. Other investigators were unable to confirm these findings by demonstration of deletions involving a similar chromosomal region in a different population of patients (Endo 1987) but Herrera's cytogenetics appeared to be sound. Since that time only two further cases of cytogenetic abnormalities have been reported in association with FAP. These cases

comprised two brothers with an interstitial deletion involving 5q21-22 (Hockey 1989). Therefore, the identification of the single case of Herrera opened the way for detailed study of the region using genetic linkage analysis with polymorphic DNA markers.

1.2.3 Genetic linkage analysis and the localisation of *APC* to chromosome 5q21-22.

Following Herrera's report of a chromosome 5q deletion in a patient with FAP, two groups, in London and in Utah, joined in a race to establish, or to refute, genetic linkage of the gene for FAP to polymorphic DNA markers known to map to that chromosomal region. In August 1987 Bodmer and colleagues published the first report establishing the localisation of APC to 5q21-22 by demonstrating genetic linkage to the polymorphic DNA probe C11P11 (Bodmer 1987). C11P11 was an anonymous DNA fragment which had been cloned as part of a random search for probes recognising DNA restriction fragment length polymorphisms (RFLP) and the existence of such a marker in the region allowed rapid regional localisation of *APC*.

Bodmer detected no recombinants between *APC* and C11P11 and suggested that C11P11 was very close to the *APC* gene indeed. However, there was no evidence of linkage disequilibrium and the lod score was only 3.26 for linkage of C11P11 to APC, resulting in wide 95% probability limits. Localisation of C11P11 by in situ hybridisation placed the APC gene in the chromosomal bands 5q21-22 (Bodmer 1987). Shortly after the establishment of primary genetic linkage in FAP by Bodmer, the Utah group also reported linkage of APC to C11P11 (Leppert 1987). Again no recombinants were detected between C11P11 and APC in this study and the combined lod scores from London and Utah were 6.63 and so strongly supported the localisation of APC close to C11P11 at 5q21-22.

Regional localisation was only the first step in isolation of the *APC* gene itself and so a high density genetic and physical map of the region was now required. However with such information as to the whereabouts of *APC*, it was then possible to examine the status of chromosome 5 in malignant tissue from sporadic colorectal cancers.

1.2.4 Chromosome 5 allele losses in sporadic colorectal carcinomas suggesting *APC* tumour suppressor activity.

Loss of genetic material can be detected by cytogenetic analysis when relatively large chromosomal fragments are involved. Sub-microscopic losses can be detected by demonstration of loss of DNA marker probes known to map to that region.

Tumour karyology in colorectal cancer is technically extremely difficult, both in the preparation of chromosome spreads and in reading the widely aberrant, hyperdiploid karyotypes which result. However, one report identified chromosome 5 as frequently structurally abnormal, although many other aberrations were also described (Reichmann 1981). In addition, a specific interstitial deletion of the long arm of chromosome 5 was reported in one tumour karyotype (Ferti-Passantonopoulou 1986). Notwithstanding the technical difficulties of tumour karyology, these findings combined with the localisation of the APC gene on the long arm of chromosome 5 strongly suggest a possible tumour suppressing activity for APC.

Loss of genetic material from 5q was more critically assessed by the use of polymorphic chromosome 5q DNA markers (Solomon 1987). The probe lambda MS8 which recognises a hypervariable (mini-satellite) region near the tip of chromosome 5 at 5q35, and another polymorphic DNA marker L1.4, were used to search for loss of constitutional heterozygosity. Demonstration of such allele loss would indicate by inference that the APC gene was also lost. Solomon found that up to 40% of sporadic colorectal cancers exhibited loss of genetic material from chromosome 5 by this approach.

The cytogenetic findings and the allele loss study of Solomon indicate that loss of the region of chromosome 5 including *APC* appears to occur in a non-random fashion in colorectal cancer tissue. The two mutation hypothesis requires that both alleles of a tumour suppressor gene are inactivated. The detection of substantial loss of genetic material from the long arm of chromosome 5 on which the gene for FAP had been localised strongly supported a recessive determinism, or tumour suppressing function, for *APC*. The assumption is made that the remaining *APC* allele is inactivated by a mechanism such as a very small deletion or perhaps a point mutation and so the chromosome 5 loss in the cancer tissue serves to unmask a mutant gene on the remaining chromosomal homologue.

It is important to note that loss of *APC* can only be inferred from these studies and the specific involvement of *APC* was still to be confirmed. Notwithstanding this, these findings are strongly supportive of the proposal that the gene for Familial Adenomatous Polyposis is indeed a tumour suppressor gene.

1.3 Statistical and epidemiological support for the existence of tumour suppressor genes

This section reviews the evidence for tumour suppressor genes from statistical analysis of the epidemiology of the rare heritable childhood cancer

syndromes, especially retinoblastoma. However there is also evidence from the epidemiology of common adult cancers that a two mutation, or possible recessive determinism compatible with homozygous inactivation of putative tumour suppressor genes, is operating at least as a rate limiting step in some of the common human malignancies.

1.3.1 Statistical analysis of the epidemiology of retinoblastoma.

Retinoblastoma is an uncommon tumour of childhood and the disease can occur in sporadic form, where there is no family history, or as a inherited familial trait. Familial and sporadic cases are identical pathologically but familial cases occur at an earlier age and the tumours are usually multiple and bilateral, whereas sporadic cases occur at a later age and the tumours tend to be unilateral. Knudson noted that the germline mutation was not sufficient in itself for tumour development since some obligate gene carriers never developed tumours but did pass on the mutant gene to their offspring (Knudson 1971). The numbers of tumours developing in the eyes of familial cases were counted and shown to fit a Poisson distribution (Knudson 1971), suggesting a random event which Knudson proposed to be a somatic second mutation. When the incidence rate of tumours was plotted on a logarithmic axis against age, there was a curvilinear decline of tumour development in the sporadic, unilateral, cases. In contrast, incidence in bilateral (ie familial) cases declined with age in a linear fashion on the semilogarithmic plot. This was powerful evidence to support a two mutation theory for retinoblastoma, thereby inferring a recessive mode of action with mutations requiring to inactivate the retinoblastoma gene for the development of tumours. Implicit in this two hit hypothesis is the notion that mutations of the same gene occur in both the familial and the sporadic forms of cancer. It is important to note that these calculations are mathematical derivations designed to fit a hypothesis and cannot exclude possibilities which have not yet been considered. Such a hypothesis based on mathematical considerations clearly requires substantiation from biological observation.

Knudson's seminal statistical analysis of the epidemiology of retinoblastoma has stood the test of time and has been extensively reviewed by him (eg. Knudson 1985, Knudson 1989). Subsequent molecular genetic analysis has confirmed this two-hit hypothesis. However it was 14 years prior to Knudson's original paper that a two mutation theory of carcinogenesis was first shown to hold for many common adult cancers.

1.3.2 Epidemiological analysis in the common adult cancers.

Epidemiological support for a two-hit theory of carcinogenesis has been evident for many years. Armitage and Doll are widely quoted as having demonstrated the involvement of several mutational steps in carcinogenesis when they assumed a variable level of environmental carcinogen (Armitage 1954). However, Platt suggested that only two mutations might be involved in carcinogenesis (Platt 1955). The first mutation would allow clonal proliferation and a growth advantage predisposing that clone to a second mutation which would induce malignancy. Armitage and Doll re-examined their original data but in this analysis assumed a constant exposure to carcinogen (Armitage 1957). This analysis favoured a two-mutation hypothesis for carcinogenesis and appeared to account for the observed incidence of many of the common adult cancers, notably breast and colon cancer. Ashley also proposed that cancer could arise via two different physiological stages but calculated that 3-7 mutations were involved in the genesis of gastric cancer in females (Ashley 1969). He also proposed that the multiple hit and the two hit theories were not mutually exclusive.

Current knowledge of the involvement of activating mutations in oncogenes and as well as the inactivation of both alleles of recessive tumour suppressor genes clearly cannot be explained on the basis of only two mutations. However a two-step theory of mutagenesis would fit for the critical rate-limiting steps along the pathway to selection for a malignant clone (Knudson 1989). Events at other loci may confer further growth advantage or promote tumour progression and metastasis . The initiating mutation may render the proliferating clone more susceptible to such subsequent mutations.

It is still too early to be sure that common adult cancers are indeed rate-limited by the need for two inactivating mutations at the same tumour suppressor locus. However it is tempting to believe that the paradigm of retinoblastoma holds not only for embryonal tumours but also for colorectal cancer and other common malignancies.

1.4 Experimental evidence for tumour suppressor genes

1.4.1 Evidence from somatic cell hybrids.

Early experiments utilising somatic cell hybridisation techniques suggested that cancer genes behaved in a dominant fashion. When cells of a low malignant potential were fused with those of high malignant potential, the resultant hybrid remained of the same phenotype as the most malignant of the parent cells (Barski

1962, Scaletta 1965). However these findings were contradicted by evidence for tumour suppressing effects of normal or wild-type genes.

The discovery that certain 'cancer' genes are recessive was first demonstrated by the cell fusion experiments of Harris and Klein (Harris 1969a, Harris 1969b). Fusion of normal mouse fibroblasts with a tumorigenic cell line resulted in extinction of tumorigenicity in the resultant hybrids so long as specific chromosomes were not lost. The expulsion of certain chromosomes resulted in the reappearance of the tumorigenic phenotype. Harris first suggested that loss of only a single chromosome might be sufficient for the re-emergence of the malignant phenotype (Harris 1969a). This has subsequently been confirmed in at least some crosses and the particular involvement of mouse chromosome 4, homologous to human chromosome 1, has been demonstrated (Jonasson 1977). Specific loss of human chromosome 11 from a normal-malignant human-human fusion hybrid as demonstrated by allele loss of a polymorphic chromosome 11 DNA marker has also been shown to result in the reappearance of the malignant phenotype (Stanbridge 1981).

The involvement of chromosome 11 has been investigated further by an elegant experiment in which a single normal human chromosome 11 was introduced into a tumorigenic HeLa-human fibroblast hybrid (which lacked chromosome 11). This resulted in suppression of the malignant phenotype (Saxon 1986). This work was taken further by the introduction of a single chromosome 11 into a tumorigenic Wilms' tumour cell line which resulted in reversion to a non-tumorigenic phenotype (Weissman 1987). This is powerful evidence to support the notion of a recessive mode of action of tumour suppressing genes and is consistent with the view that inactivating mutations within these genes can result in a heritable cancer syndrome.

A recent study from Stanbridge's group has demonstrated another specific chromosome capable of tumour suppression in a tumorigenicity assay. A normal human chromosome 6 was introduced into a tumorigenic human melanoma cell line and suppression of tumorigenicity was demonstrated (Trent 1990). The reason for selection of chromosome 6 for such a study was that human melanoma tumour tissue has been shown to have consistent karyotypic abnormalities involving chromosome 6q. Thus this is another example of tumour suppressor loci being identified by tumour cytogenetics (see section 1.4.4 and 1.4.5).

Studies involving human intraspecies hybrids have suggested tumour suppressor activity on many chromosomes such as chromosomes 1,2,4,11,13,17 and 20 (Stanbridge 1988). It is likely that inactivation of any one or a number of these genes might be involved in the genesis of particular tumour or in individual steps towards the malignant phenotype in a particular class of malignancy.

However deletion of certain genes may well be a common molecular end point in the biology of progression and metastasis of many tumour types.

The ability of genetic material from normal cells to suppress malignancy sometimes requires multiple copies of a particular chromosome to have an effect (Evans 1982) and the ratio of malignant-derived chromosomes to normal chromosomes is important (Harris 1988). Therefore it is seems that for at least some tumour suppressor genes there is a gene dosage effect.

The mechanism of suppression of tumorigenicity by normal genetic material is now becoming more clearly understood. Comings (Comings 1973) proposed that genes active during embryonic development are suppressed and the target cell is induced to differentiate by a controlling or suppressing gene. This remarkable piece of insight now appears to be well founded. Knudson also proposed a balance between genes promoting proliferation and those regulating growth and inducing differentiation (Knudson 1985). He originally coined the term antioncogene to indicate the relationship with dominant oncogenes but the term 'tumour suppressor gene' is now preferred. Recently, direct experimental evidence has been published which demonstrates that putative tumour suppressor loci do indeed exert control over cells exhibiting excessive proliferation and which have lost a degree of differentiation. When a malignant cell line is crossed with a normal fibroblast, and tumour suppression is achieved, the non-tumorigenic hybrids have been induced to undergo terminal differentiation, have histological features of fibrocytes and even produce collagen (Harris 1985, Stanbridge 1981).

The dominant cellular transforming genes or oncogenes (Cooper 1982) have now been shown to have diverse functions but appear to play a role in cellular proliferation. Mutational activation of an oncogene could lead to uncontrolled proliferation and de-differentiation. Hence the hypothesis of Comings may apply to tumour suppressor genes which could be the natural regulators of oncogenes. The interaction between the two may be unbalanced by either mutational activation of the oncogene or inactivation of the tumour suppressor gene, or perhaps even by a combination of both.

1.4.2 Somatic cell hybrids and tumour suppressing effects of specific chromosomal loci.

A great deal of normal genetic material is transferred by cell fusion techniques and the chromosome complement of the resultant fusion hybrid tends to be unstable. Even when a single whole chromosome is introduced into a tumorigenic cell line and tumour suppression results, the amount of DNA being transferred is large. Therefore identification of any particular locus with onco-

suppressor activity by this method alone will be extremely difficult, isolation of the gene involved and analysis of its function even more so.

The ultimate test of the tumour suppressing activity of any chromosomal locus is the reversion of a tumorigenic phenotype in a cell line by introduction of a single putative tumour suppressor gene. This has been achieved for the retinoblastoma gene and this is discussed below.

1.4.3 The paradigms of retinoblastoma and Wilms' tumour

The gene responsible for retinoblastoma stands out as a paradigm for all putative tumour suppressor genes. As discussed above, epidemiological analysis had predicted a requirement for two hits at the same gene locus for tumour development. This was followed by cytogenetic analysis which demonstrated visible constitutional deletions of chromosome 13 which always involved the region 13q14 (Yunis 1978). More than 30 cytogenetic deletions have now been reported and all involve the same chromosomal region (Hansen 1988). A gene close by on chromosome 13q, the esterase D gene, was shown to be close to the putative Rb gene in genetic linkage studies (Sparkes 1983). Shortly afterwards retinoblastoma tumour tissue was shown to have lost genetic material specifically from chromosome 13q involving the esterase D gene by analysis using RFLP DNA markers (Cavenee 1983). Loss of heterozygosity occurred in more 73% of tumours (Hansen 1988). Most important for support of the two hit hypothesis was the discovery that, in familial cases, the allele which was retained in the retinoblastoma tumour tissue was the mutant one inherited from the affected parent (Cavenee 1985). Subsequently, 2 tumours of the 37 examined in one study exhibited homozygous loss of a tightly linked RFLP marker (Dryja 1986). These deletions occurred in the germline or as somatic mutations (Dryja 1986). These findings suggest that although a germline mutation is heterozygous, when a subsequent genetic event inactivates the remaining retinoblastoma gene allele in the retinocyte, the result is of homozygous loss of that gene function. Two somatic inactivating mutations could have the same net effect, conferring growth advantage to that retinal epithelial cell and resulting in expansion of that (now neoplastic) clone. The probe found to show homozygous deletions was then used to isolate fragments of DNA from the surrounding region and the resultant clones tested for the hallmarks of a transcribed gene and in particular for the likely traits of the putative retinoblastoma tumour suppressor gene. The gene (known as Rb-1) was cloned shortly afterwards (Friend 1986) and so the tumour suppressing activity of this gene could then be put to the ultimate test. Transfection of the cloned Rb-1 gene into a tumorigenic retinoblastoma cell line using a retroviral vector has recently been achieved and this

has shown suppression of the malignant phenotype (Huang 1988). This is the first direct evidence which demonstrates tumour suppressing activity for the retinoblastoma gene and lends considerable weight to the notion of recessive tumour suppressing genes in general.

The function of the Rb-1 gene remains to be fully elucidated but the process of identification of this gene has proven invaluable in work on other tumour suppressor genes. Clearly, this work confirmed that two hits were required at the same gene locus, resulting in homozygous inactivation of the Rb-1 gene and two somatic mutations can have the same effect as one germline and one somatic event.

Another embryonal cancer, Wilms' tumour, serves to introduce complexity into the field, in contrast to the relatively straightforward case of retinoblastoma and the explanation of tumour suppressor genes based on that paradigm. It was predicted that Wilms' tumour was also due to two events required to inactivate both alleles of the same gene and that these events were at the same locus in the familial and sporadic forms of the disease (Knudson 1972). Cytogenetic studies demonstrated a translocation involving chromosome 11 (Ladda 1974) and several deletions have now been described which all involve 11p13 (Knudson 1985). RFLP markers have demonstrated loss of genetic material from the same region (Koufos 1984) but genetic linkage analysis in the rare familial type of Wilms' tumour has excluded the region 11p13 as that carrying the heritable form of the disease (Grundy 1988, Huff 1988). A further complication is the association of the Beckwith-Wiedemann syndrome which predisposes to Wilms' tumour. The gene responsible appears to map to chromosome 11p15 and clearly does not involve the Wilms'/aniridia locus (Scrable 1987). Notwithstanding these complexities, the data on Wilms' tumour do support the notion that the Wilms'/aniridia gene is indeed a tumour suppressor gene but that more than one gene is involved.

1.4.4 Cytogenetic observations in colorectal cancer.

Tumour karyology was an important pointer in helping to localise the *APC* gene (Ferti-Passantonopoulou 1986). A number of other possible tumour suppressor loci involved in colorectal carcinogenesis have been indicated by tumour cytogenetics and these include chromosomes 8 and 14 (Mitelman 1974); 8,17 and 18 (Muleris 1985); 1,8p,13,17 and 18 (Reichmann 1981). The losses or structural rearrangements detected in these studies were of a non-random nature and these findings must be taken to indicate a strong possibility that tumour suppressor genes are present at these loci. There are few cytogenetic data on blood lymphocytes of colorectal cancer patients but one study has shown a constitutional chromosome 8:12 translocation which was present in the colorectal cancer tissue and was also

passed on to the patients offspring who developed a rare testicular malignancy (Pathak 1986). This is an exciting discovery since it may indicate a tumour suppressor gene involved not only in tumour biology, but also in the inheritance of cancer susceptibility.

1.4.5 Structural alterations at putative tumour suppressor genes in colorectal cancer.

Three tumour suppressor loci have now been well characterised in colorectal carcinogenesis, the gene for p53 on chromosome 17p, the DCC gene on chromosome 18q and the *APC* gene on chromosome 5 to which this thesis is addressed. Discussion of the experimental work presented in this thesis and its relevance to recent findings relating to the role of *APC* in familial and sporadic colorectal cancer is reserved for Chapter 9. This section is restricted to discussion of the involvement of putative tumour suppressor genes on chromosomes 17 and 18.

The gene for p53 on chromosome 17p

Tumour cytogenetics had indicated a possible recessive colorectal cancer gene on the short arm of chromosome 17 (Muleris 1985, Reichmann 1981) but the first molecular genetic evidence for a recessive tumour suppressor locus on chromosome 17p (Fearon 1987) was the demonstration of clonal loss of genetic material at a highly polymorphic locus YNZ22 which mapped to the short arm of chromosome 17 (Nakamura 1987) in a high frequency of colorectal cancers. The frequency of YNZ22 allele loss in the cancers was 76% while the frequency in adenomas at the same locus was only 3%. Fearon was also able to show that, in carcinomas which exhibited allele loss and which had a benign element adjacent from which the malignancy would be presumed to have arisen, 2 out of the 3 benign tumours had not lost heterozygosity. This work suggested that not only was there a tumour suppressor locus on chromosome 17p, but also that it was involved in the biology of the malignant but not the benign neoplasm.

The findings of 17p allele losses in colorectal cancer with a reduced frequency in colorectal adenomas have been confirmed by many investigators (Baker 1989, Delattre 1989, Fey 1989, Law 1988, Lothe 1988, Monpezat 1988, Vogelstein 1988, Vogelstein 1989). Notably 17p allele losses were also noted in colorectal cancer arising in patients with FAP (Sasaki 1989). The region of chromosome 17p which was consistently deleted in colorectal cancers included the gene which codes for a protein, p53, (Baker 1989) which had been implicated as an oncogene capable of inducing cellular transformation or at least of enhancing the effect of other transforming genes such as *ras* (Editorial 1988). Baker demonstrated that in

colorectal cancers which had lost one p53 allele, the retained allele was mutant. Baker suggests that p53 interacts with other agents to result in suppression of neoplastic change. Mutant p53 may compete with normal p53 for the binding sites required for tumour suppression and loss of the normal allele by structural deletion would produce a more pronounced effect. Thus p53 seems to have both tumour suppressing and oncogenic activity, depending on whether it is mutant or not. The tumour suppressing nature of non-mutant p53 was confirmed later that year when it was shown to inhibit oncogene-induced cellular transformation (Finlay 1989). The function of p53 has not been fully elucidated but wild type p53 has been found bound to transforming proteins from oncogenic viruses (Editorial 1988) and Baker has suggested that the tumour suppressing capability of p53 is due to its ability to block virus-associated neoplastic change (Baker 1989). Increased levels of mutant p53 also has been demonstrated in lung cancer (Iggo 1990) and it may be that p53 is involved in many different cancers. The work on p53 is at an exciting stage. The biological function of the first cloned and sequenced gene with demonstrable tumour suppressor activity can now be elucidated. There is also the possibility of targeting tumour cells *in vivo* by designing monoclonal antibodies to mutant p53 in order to deliver chemotherapeutic agents or radioisotopes for imaging or treatment of cancer.

The DCC gene on chromosome 18q

In a similar manner to chromosome 17p, tumour karyology suggested that there may be a recessive gene or genes on chromosome 18 involved in the biology of colorectal cancer (Muleris 1985, Reichmann 1981). This has been supported by allele loss studies which show chromosome 18 deletions in almost 80% of colorectal cancers (Delattre 1989, Law 1988, Monpezat 1988, Vogelstein 1988, Vogelstein 1989) and a similar frequency of allele loss is seen in FAP carcinomas (Sasaki 1989). In contrast to the situation with the gene for p53 on chromosome 17p, there was no candidate target gene on chromosome 18q. Hence, identification and sequencing of the responsible gene was required. This has been achieved by an elegant series of experiments by Vogelstein and his colleagues (Fearon 1990). A common region of deletion in colorectal cancer tissue had been identified (Vogelstein 1988) and the breakpoint of a rearrangement in a single cancer was cloned. From this point a massive *tour de force* of chromosomal walking has resulted in cloning of a 1.2 megabase gene with a 12kb mRNA which is now almost completely sequenced (Vogelstein personal communication 1990). The gene has been called the DCC (deleted in colon cancer) gene and somatic mutation have been identified within it at an apparent mutational 'hot-spot'. Expression of DCC has been demonstrated in a number of tissues, including colonic mucosa and greatly reduced or absent in

colorectal cancer tissue. This, combined with the finding of a homozygous intragenic deletion is powerful evidence to suggest that DCC is indeed another tumour suppressor gene. Preliminary DCC amino acid sequence data suggest similarity to known cell-adhesion molecules. Therefore DCC may play a role in cell to cell interactions, an attractive concept in view of the well-known derangement of normal growth inhibition in cancer.

There is little doubt that DCC is involved in the biology of colorectal cancer but another exciting aspect of the involvement of DCC in colorectal carcinogenesis is the possibility that it may be involved in the inheritance of colorectal cancer susceptibility. Tentative genetic linkage to the Kidd blood group was established in one family with Cancer Family Syndrome by Lynch and colleagues in 1985 (Lynch 1985b). At that time the Kidd blood group locus (JK) was believed to map to chromosome 2. However, JK has been reassigned to chromosome 18 (Geitvik 1987) and with it the possible CFS locus (Boman 1988). Recently genetic linkage has been demonstrated between JK and polymorphic RFLP marker from within DCC (Vogelstein personal communication 1990). These extremely important findings may represent the first steps towards identification of a blood test to identify individuals at high risk of colorectal cancer such that screening and prophylaxis could be undertaken to reduce overall mortality from the disease.

Other potential tumour suppressor loci

It is likely that there are tumour suppressor loci at other chromosomal locations and these are indicated by non-random allele losses and rearrangements at a number of loci such as chromosomes 6, 8, 12, 14 and 22 (Sasaki 1989, Thein 1987, Vogelstein 1989). Some of these may represent genes involved in different aspects of malignancy such as invasion and metastasis.

This is an exciting time in fundamental understanding of colorectal cancer with particular regard to tumour suppressor genes and there is reason to be optimistic that research will make real improvements to the burden of colorectal cancer in the world population. Abnormalities of tumour suppressor genes are not the only genetic alterations described in colorectal cancer and some of these other changes are detailed below.

1.5 Genetic changes in colorectal cancer which do not involve aberrations of putative tumour suppressor gene loci.

This section briefly reviews the genetic changes noted in colorectal carcinoma other than changes to putative tumour suppressor genes. It is not intended to be an exhaustive review since the main theme of this work was an analysis of the involvement of the *APC* gene in colorectal carcinogenesis.

1.5.1 DNA methylation status in colorectal cancer.

Methylation of DNA is a structural modification mainly of the cytosine-guanine dinucleotides in the mammalian genome. The majority of cytosine residues are methylated in normal, fully differentiated cells (Razin 1980). A decrease in the level of methylation appears to be associated with gene activation (Razin 1980).

Since DNA methylation status is correlated with differentiation and with gene activation, and colorectal cancer tissue is abnormal in both of these respects, Goelz examined DNA methylation status in colorectal adenomas and carcinomas (Goelz 1985). Using methylation-sensitive restriction endonucleases (Hpa II and Hha 1) which cut at CG residues, Goelz examined the methylation status of a total of 10 genes at random by probing genomic Southern blots from benign and malignant colorectal neoplasms with the respective gene probe. The endonuclease Msp.1 served as a control for Hpa II since it cleaves at the same CCGG recognition site regardless of the methylation status of the internal C residue. The methylation status of the adjacent normal tissue served as a tissue control.

Goelz found that 4 genes in particular of the 10 studied were significantly hypomethylated in all 23 neoplasms examined and that adenomas and carcinomas were hypomethylated to a similar degree. The genes involved were growth hormone, gamma-globin, α-chorionic gonadotropin and gamma-crystallin. These genes are not thought to be specifically involved in carcinogenesis but this work implies and overall activation of genes, including those involved in the neoplastic process. It was also noted that the changes were quite heterogeneous in that different sites of methylation occurred in the same tumour types arising in different individuals. Goelz proposed that hypomethylation could lead to expression of dormant genes in the colonic epithelium which might then confer growth advantage.

Hypomethylation might also interfere with chromosomal condensation and subsequent reassortment at the time of mitosis. This might then lead to chromosomal breakages and it is tempting to postulate that loss of specific tumour suppressor loci by this mechanism would then confer a selective growth advantage and the continuation of that abnormal clone.

Little work appears to have been carried out on DNA methylation status in colorectal carcinogenesis since the work of Goelz. However, DNA hypomethylation does appear to be an early event and certainly precedes malignant transformation. Whether DNA hypomethylation is a cause or an effect of neoplasia remains to be elucidated.

1.5.2 Flow cytometry and ploidy levels in colorectal cancer.

Tumour cytogenetic analysis has demonstrated that colorectal cancer cells are seldom, if ever, diploid for every chromosome (Ferti-Passantonopoulou 1986, Mitelman 1974, Muleris 1985, Pathak 1986, Reichmann 1981). However, tumour cytogenetics is laborious and is best suited to the examination of specific chromosomal abnormalities. A more global examination of the amount of DNA (ploidy level) in colorectal cancer tissue can be undertaken by analysis of the total nuclear DNA content using flow cytometry. Tumours vary in the degree of abnormality of DNA level (aneuploidy) which they exhibit and this can be compared to the biological behaviour of the tumour.

In various studies, the ploidy level of colorectal cancer has been shown to distinguish two populations of tumours; those that are severely aneuploid and those that are predominantly diploid. The predominantly diploid tumours may well include a minority of cells which are aneuploid but, overall, the main population tend to be diploid and hence 'mask' the minority. The aneuploid group comprise around 40%-55% of all tumours (Armitage 1985, Jones 1988, Wolley 1982). There is no correlation with the presence of aneuploidy and tumour histological grade or pathological stage. There are conflicting data on the relationship of ploidy level and survival. In some studies, the presence of aneuploidy in colorectal cancer is highly significantly correlated with poor survival (Armitage 1985, Wolley 1982). In one of these studies, the 5 year survival for patients who had a diploid tumour resected was 43% compared with 19% for those patients with an aneuploid tumour (Armitage 1985). However, there is evidence that DNA ploidy status confers no independent prognostic value after resectibility, pathological classification and patient age (Jones 1988). It has been shown that DNA diploid rectal carcinomas are less likely to recur locally after surgery and adjuvant radiotherapy and DNA ploidy seems predict the response to adjuvant radiotherapy in these tumours (Jones 1989). Careful, long-term follow-up of prospectively collected patient populations will be required to clarify the situation.

DNA aneuploidy has been demonstrated to be less frequent in colorectal adenomas than in carcinomas (Quirke 1986). Quirke found that 6% of adenomas were aneuploid and this was significantly associated with size of the adenoma.

Since tumour size has been shown to be a strong predictor of subsequent malignant transformation (Morson 1983, Muto 1975), this supports the notion that aneuploidy is associated with aggression. Quirke also demonstrated that carcinomas with synchronous adenomas tended to show aneuploidy less frequently than those carcinomas without synchronous adenomas. This suggests that the tumours with associated adenomas may be a less aggressive sub-group of colorectal cancers.

The mechanism by which aneuploidy occurs is unknown, but one hypothesis suggests that it occurs due to a failure to reassort chromosomes correctly at mitosis (Nowell 1986, Wyllie 1989). This would allow the loss or gain of any chromosomal loci postulated to be important in the pathogenesis of colorectal cancer. Those clones with a growth advantage would then be subject to positive selection. Thus a mechanism for loss of tumour suppressor genes would be embraced by such a process. This scheme would also concur with that postulated by Goelz (Goelz 1985). Aneuploidy may be due to abnormalities of DNA methylation status or of genes, such as p53, which may influence spindle formation and result in disruption correct chromosomal reassortment.

1.5.3 Oncogene alterations.

This section briefly describes the nature of oncogenes in general and indicates those reported to have a specific involvement in colorectal carcinogenesis. The main theme of this thesis is concentrated on tumour suppressor genes with particular reference to *APC*. The purpose of this section is to give an overview of this class of genes whose action is believed to require the modulating effect of tumour suppressor genes for the maintenance of a natural balance of cellular activity and proliferation (Comings 1973, Knudson 1985).

Oncogenes are genetic elements which can initiate and/or maintain a transformed phenotype in a cell and are thus said to act dominantly. There are now over 40 known oncogenes. In addition to classical *in vitro* transformation assays using mouse NIH 3T3 cells, these genes were found to be capable of transforming rodent and human cells into cells which form tumours *in vivo* (Cooper 1982, Bishop 1985).

The native human oncogenes, or proto-oncogenes are involved in normal cellular activity, particularly those functions associated with cellular proliferation. The precise functions of these genes are diverse; they include growth factors, growth factor receptors, signal transducers, protein kinases and transcriptional activators (Bishop 1985, Land 1984). Many proto-oncogene functions appear to promote cell growth and proliferation. It is abnormal expression, or activation, of these genes which has been shown to account for their characteristic transforming properties

(Bishop 1985, Cooper 1982, Duesberg 1985, Slamon 1984). There are several ways that a proto-oncogene can be converted into a transforming oncogene which will promote excessive cellular proliferation and some examples are given here.

A non-mutant proto-oncogene may be expressed in an abnormal, unregulated way by translocation to a site without an oncogene regulator region, as in the case of the involvement of the *c-myc* oncogene in Burkitts lymphoma. The *myc* proto-oncogene is normally located on chromosome 8 but Burkitts lymphoma is associated with a chromosome 8:2, 8:14 or 8:22 translocation which brings the *myc* locus into close proximity with the immunoglobulin enhancer sequences. In some instances the expressed sequences of *myc* itself can be removed from the regulatory influence of its own untranslated first exon. The net result for both of these mechanisms is overexpression of *myc* gene product. Oncogenes can also be activated by gene amplification (duplication) as in the case of N-*myc* which is found in multiple copies (up to 300) in around 50% of neuroblastomas and this is associated with a poor prognosis (Weber 1987).

Another mechanism for conversion of an oncogene into a transforming gene is by mutation within the gene itself. Although the *ras* gene had been shown to induce a transformed phenotype in NIH 3T3 transformation assays (Bishop 1985, Duesberg 1985, Land 1983), it was not initially realised that the gene resulting in transformation was a mutant form of the *ras* proto-oncogene (Cooper 1982). However, it was later shown that activation of *ras* was due to a point mutation (Tabin 1982) and that two different point mutations at the 12th codon could both activate the gene (Capon 1983). The result of such mutations is to substitute a valine or cysteine for the normal glycine found at codon 12 in the *ras* gene product (p21). Mutations at codons 13 and 61 are also now well described and appear to have the same functional effect (Barbacid 1987) as a codon 12 mutation. The three amino acids coded by these sites all reside in the GTP binding region of the p21 product. The result is that *ras* appears to be 'switched on' in an uncontrolled fashion and causes inappropriate signals at the membrane for the cell to keep cycling (Bishop 1985).

Oncogene abnormalities which have been reported to be involved in colorectal carcinogenesis include abnormal levels of expression of *fos*, *myc*, *ras*^{Ha} and *ras*^{Ki} (Slamon 1984). However, the role of *fos* involvement is disputed (Alexander 1986). In addition, the *src* oncogene protein product (pp60) has been demonstrated in abnormal amounts in colorectal cancer tissue (Bolen 1987). Amplification of *myc* has been demonstrated (Alexander 1986) and elevated levels of c-*myc* mRNA and protein product have also been demonstrated (Erisman 1985). However it is not clear whether myc expression merely reflects proliferative activity

rather than influences it because no amplification or activating mutations have been detected even in cancers which are over-expressing myc (Erisman 1985).

The family of *ras* oncogenes is the most extensively studied group of transforming genes in colorectal cancer. Elevated expression of the *ras* p21 protein product has been shown to occur in 100% of colorectal cancers in one study (Hand 1987). Over-expression of the *ras* mRNA (Spandidos 1984) and of the p21 protein product (Williams 1985) has been demonstrated in both colorectal adenomas and carcinomas, suggesting that *ras* activation may be an early event in colorectal carcinogenesis. This is supported by the finding that, although p21 expression was enhanced in primary carcinomas above that in adjacent normal colorectal mucosa, it was relatively lower in more pathologically advanced cancers and expression was lower in distant colorectal metastases than in the primary tumour (Gallick 1985). The mechanism of *ras* activation in colorectal neoplasia is most frequently by mutations in the Ki *ras* oncogene (Vogelstein 1988). The point mutations involved can occur at codons 12, 13 and 61 of the *ras* gene but codon 12 lesions are the most common (Bos 1987). Ki *ras* mutations have been demonstrated in around 40% of colorectal cancers (Bos 1987, Delattre 1989, Forrester 1987, Vogelstein 1988). One of the most striking findings is that mutational activation can precede the development of malignancy since mutations have been detected in adenomas (Bos 1987, Forrester 1987, Vogelstein 1988) and the frequency of detection of Ki *ras* mutations is highest in adenomatous tissue within a carcinoma (Bos 1987, Vogelstein 1988). This is compelling evidence to suggest the early involvement of Ki ras mutations in colorectal carcinogenesis. The frequency of Ki ras mutations in adenomas from FAP patients has been reported by various authors as 7% (Farr 1988), 13% (Sasaki 1990) and 13% (Vogelstein 1988) which is substantially lower than that seen in sporadic adenomas. However most of these lesions were small and of low grade dysplasia and as will be discussed in Chapter 6 the malignant potential of any one FAP adenoma is low given the hundreds of lesions present in the colon of affected patients. The frequency of Ki ras mutations in carcinomas arising in patients with FAP is 36% which is very similar to that seen in sporadic cases (Sasaki 1990). The published data suggest that mutation of Ki ras is not involved in the genesis of adenomatous change but is involved at the progression of adenoma to carcinoma.

The role of activated oncogenes in colorectal cancer is now becoming more clear but the interaction such genes and their regulators, which may well be a class of tumour suppressor genes, remains to be elucidated.

1.6 Aims of the project

The central theme of this project was to extend knowledge of the *APC* gene, its role in the inheritance of Familial Adenomatous Polyposis and its putative role as a tumour suppressor gene in sporadic colorectal carcinogenesis.

Following regional localisation of APC to chromosome 5q21-22 just prior to the start of this work, the aims of the project were to construct a high resolution genetic linkage map of the chromosomal region around the gene in family studies of kindreds affected by FAP. It would then be possible to ascertain whether the inherited mutation was sufficient for the development of FAP adenomas or whether a further event at that locus was required. The map would also allow examination of the nature, extent and APC specificity of the loss of genetic material from chromosome 5 reported in sporadic colorectal carcinomas. The stage of tumorigenesis at which chromosome 5 deletion occurs could then be examined by a search for deletions in sporadic colorectal adenomas. The most immediately clinically relevant aim was to assess the value and the validity of linkage analysis derived risk estimation for presymptomatic diagnosis of Familial Adenomatous Polyposis in at-risk family members.

1.7 Experimental approach

A substantial clinical resource was collected for molecular genetic analysis and includes blood samples from patients with colorectal adenomas and carcinomas and members of FAP families, in addition to stockpiling tumour tissue from such lesions. One affected member of each family has been karyotyped in the hope of detecting an invaluable translocation.

The identification and complete ascertainment of kindreds affected with Familial Adenomatous Polyposis required to be initiated as there was no Polyposis Registry in Scotland. The kindreds were fully ascertained by myself and Chapter 3 describes the results of screening of the family members for the presence of polyps and extra-colonic features of the syndrome.

Chapter 4 presents the results of genetic linkage analysis in FAP kindreds using DNA probes which recognise polymorphisms on chromosome 5 in the region thought to harbour the APC gene. A genetic linkage map of the region around the APC gene was constructed and Chapter 5 presents the application of this map and other RFLP markers spanning chromosome 5 to deletion analysis in a large number of sporadic colorectal carcinomas. A similar analysis of allele loss applied to familial and sporadic adenomas is presented in Chapter 6.

Genetic linkage and deletion analysis strongly suggested one of the markers might be very close to the APC gene itself. In view of this evidence, a search for constitutional rearrangements of large DNA fragments which included this locus was carried out by analysis with pulse field gel electrophoresis. This is presented in Chapter 7.

The use of DNA analysis in Familial Adenomatous Polyposis in the clinical setting is described in Chapter 8. Presymptomatic diagnosis of FAP using RFLP markers mapped by genetic linkage analysis combined with deletion analysis in sporadic colorectal carcinomas was possible for a large number of the at-risk family members. The value and the validity of such presymptomatic diagnosis is presented.

CHAPTER 2

MATERIALS AND METHODS

This chapter describes the methods used for clinical and family ascertainment in addition to a description of the laboratory techniques employed. The description is necessarily general with regard to restriction enzymes and probe combinations but more specific information is given in the results chapter where each of the experiments is described.

2.1 Collection of families with Familial Adenomatous Polyposis (FAP).

The families with Familial Adenomatous Polyposis which participated in this project are all of Scottish descent and are not included in published genetic linkage studies other than those pertaining to this work. The probands of these families were largely identified through contact with local surgeons and a few via the Clinical Genetics Department at the Western General Hospital, Edinburgh. The diagnostic criteria for probands with FAP were those now universally accepted (Bussey, 1975), namely more than 100 adenomatous polyps of the colorectum. Fewer polyps were accepted as diagnostic in offspring of confirmed gene carriers. Prior to any direct patient contact, the general practitioner of every patient or nuclear family group was contacted and his/her agreement obtained. All family, clinical and paternity testing data has been strictly confidential throughout.

Genetic linkage analysis relies on accurate phenotypic and genealogical information in addition to reliable experimental data. The ascertainment of pedigree information and diagnostic classification of individuals within the families collected in this study has been exhaustive and I believe family data are wholly valid to date.

2.1.1 Ascertainment of FAP families.

Fifteen probands with FAP were identified. Eleven of these were suitable for full ascertainment and, with their families, comprise the study group. The families of these probands were ascertained by three main methods.

In some families pedigree information was already well documented but required extension and updating (particularly KMD 1, which was known to Mr.A.A. Gunn of Bangour General Hospital, West Lothian and to the MRC Human Genetics Unit since 1975). However family data and validation of diagnosis was checked in

every case from hospital and pathology records and by cross-checking with public records as detailed below.

Probands and family members were interviewed by myself to construct preliminary pedigrees but although many knew of the existence of some of their relatives, the pedigree information was generally scanty. Pedigree information from other sources was also cross-checked at the time of interviewing family members in every family.

Genealogical investigation was carried out by examination of public records of births, deaths and marriages at Register House, Edinburgh. I am most grateful to Susan Collyer and Rona de May of the Registry at the MRC Human Genetics Unit who carried out this work. Families with FAP were traced back as far as was practicable or until there was evidence that the disease had arisen as a new mutation (ie parents and siblings unaffected). Pedigree tracing prior to the instigation of a Scottish national register of births, deaths and marriages in 1852 is extremely time consuming as local Parish registers have to be examined and so no attempt was made to trace families prior to this date. Thus, within the limitations of public records information, there is no evidence that any of the 11 families which have been ascertained were in any way inter-related.

The clinical and pathology records of all individuals reputed to be affected were personally scrutinised in order to confirm the diagnosis. Where family members were identified by genealogical investigation, every attempt was made to confirm disease state. Many hospitals have a policy of destroying records which have not been required after 20 years or even sooner if the patient is deceased. In these cases attempts were made to locate pathology records. Unfortunately, due to the very long time periods in question, many of the hospitals involved are no longer in existence or have moved site. In addition, without the exact date of operation, extracting pathology records was impossible in some cases. Therefore, there were a few family members (particularly in KMD 1) where no absolute confirmation that they had FAP could be established. In these cases I have relied on death certificate information alone or supplemented by a history from some of the older family members.

The pedigrees derived from genealogical investigation for all 11 FAP kindreds are shown in Appendix A.

2.1.2 Ascertainment of families by clinical screening.

In order to ensure completeness and accuracy of ascertainment, I personally screened as many family members as possible. This proved invaluable because it allowed me to develop a rapport with the families which would not have been

possible otherwise. This has resulted in a very high proportion of the family members agreeing to help with the research aspect of the work. In addition, because I verified cases clinically who had been discharged from screening programs, several new cases were identified who had been previously mis-classified as unaffected. The clinical screening also facilitated the collection of tumour tissue for molecular analysis. The screening modalities employed are detailed in Chapter 3 but in brief, colonic screening was carried out by endoscopic and/or radiological means, the presence of CHRPE lesions was ascertained by ophthalmoscopy and clinical examination for the presence of other extra-colonic stigmata was undertaken.

2.2 Collection of clinical material and controls.

Collection of clinical material was carried out personally in almost all instances. Some blood samples were taken by the patient's own family doctor if there were great distances involved but in general I preferred personal contact to take the family history and to allow the patient to make enquiries as to the nature and possible benefits of the work.

I collected all tumour samples personally from the operating theatres at the Western General Hospital, Edinburgh and immediately transported them to the pathology department there to avoid degradation of the material. Collection of clinical material for this project has been approved by the local ethical committee.

2.2.1 Blood samples.

30ml of peripheral venous blood was drawn from FAP family members (10ml in Lithium Heparin anticoagulant for EBV transformation of B-lymphocytes and short-term culture for chromosome analysis, and a further 20ml in Potassium EDTA anticoagulant for DNA purification) and 20ml of blood was drawn from patients with sporadic colorectal neoplasms to serve as control for allele loss studies (all in Potassium EDTA for DNA purification). During transport or if there was any delay before processing, the blood in Lithium Heparin was kept at room temperature and the blood for DNA purification stored at 4°C.

2.2.2 Collection of adenomas from patients with FAP.

A total of 40 adenomas were collected from 4 patients with FAP at the time of prophylactic colectomy. The lesions were removed in the operating theatre side-room as soon as the specimen was available. The macroscopic examination and removal of polyps was carried out by a pathologist (Dr. AH Wyllie). The colectomy

specimen was opened on a bed of ice and cold isotonic saline poured over the mucosa to avoid drying. The polyps were removed avoiding any of the surrounding normal mucosa, placed in Eppendorf vials, snap frozen in liquid nitrogen and then stored at -70°C until ready for DNA extraction.

Histological confirmation of adenomatous change was carried out in several polyps from each case and assessment of all other similar lesions by a pathologist (Dr. AH Wyllie) confirmed the diagnosis.

2.2.3 Collection of sporadic neoplasms.

A total of 51 nonpolyposis colorectal carcinomas were collected from 49 patients (26 male and 23 female) who underwent colectomy. Twenty adenomas were harvested from 14 colectomy specimens removed for coexisting carcinoma. The resected colonic specimens were transported on ice and examined in the fresh state by a pathologist as part of the normal clinical diagnostic procedure. Histopathological assessment was carried out on a portion of tumour immediately adjacent to the sample taken for DNA analysis. All sporadic adenomas were obtained from colectomy specimens carried out for symptoms or due to the co-existence of a colorectal malignancy. The tumour tissue was stored at -70°C until ready for DNA extraction.

At the same time as harvesting carcinoma and adenoma tissue from colectomy specimens, macroscopically normal colonic mucosa, dissected free from muscularis propria, was also collected from the same specimen at a point 10cm or more from the nearest tumour.

2.2.4 Collection of archival paraffin-embedded material.

Deceased members of FAP kindreds who had undergone surgical procedures were identified during pedigree ascertainment and a search was made of pathology records for paraffin-embedded archival material sent for pathology examination at the time of surgery. Blocks of tissue were collected from 11 different patients. In order to ensure that only normal tissue was analysed, histological assessment was carried out in every case prior to DNA extraction. If tumour tissue was noted and wholly normal blocks were not available for that patient, normal material was obtained by dissecting tumour tissue away from the normal remainder.

2.3 Establishment of a renewable source of DNA.

The establishment of a renewable source of DNA was of great importance to this project in order to avoid depleting irreplaceable resources of DNA, especially since many patients were old and some have died since this project began.

2.3.1 Epstein-Barr virus transformation of peripheral blood lymphocytes; storage and maintenance of EBV transformed lymphoblastoid cell lines.

The transformation and maintenance of peripheral blood lymphocytes from family members was carried out by Dr. M Steel, Liz Harvey and myself but the method used was consistent throughout.

Ten ml of fresh heparinised blood was layered onto 10ml of Ficoll Hypaque (s.g. 1.071) and centrifuged at 2500rpm for 15 mins. with 50% brake applied. The white cell interface was collected with a pastette and washed twice with PBS. After counting, 1-1.5x10^6 cells were taken for transformation and the remainder stored (in case the transformation failed) in 5% DMSO in FCS and frozen sequentially at -20°C in the freezer and -140°C in liquid nitrogen to avoid intracellular crystal formation. The cells for transformation were incubated in small glass tubes at 37°C in 5% CO2 in transformation medium comprising 1.5ml RPMI, 5% Fetal Calf Serum (FCS), 1% glutamine, 100ul cyclosporin (10ug/ml) and 100ul B95-8 supernatant. B95-8 is an Epstein-Barr virus infected marmoset lymphoid cell line infected with which is grown as a monolayer in 200ml flasks and when deprived of nutrients for 10 days infectious virus is shed into the medium (Miller 1973). The medium was changed weekly or before if a colour change from red to yellow was noted. The cells were seen to have transformed when there was clear evidence of increasing numbers, increased cell size and cell clumping. At this stage the cells were transferred to larger glass tubes or 50ml culture flasks for bulking up. Maintenance culture conditions consisted of RPMI with 5% FCS at 37°C in 5% CO2 and medium was changed weekly. After washing, at least 3x10^6 transformed cells were then split from the main culture, spun down and the pellet resuspended in storage medium (5% DMSO in FCS). The cells were then frozen sequentially at -20°C and -140°C, as for fresh lymphocytes. The remainder of the culture was bulked up until a further 1x10^7 cells were obtained and these were then spun down for DNA extraction.

2.4 Cytogenetic studies.

At least one affected member of every FAP kindred was examined by cytogenetic analysis in the hope of discovering a deletion or critical translocation with a breakpoint in the *APC* gene. Metaphase spreads were prepared by myself but chromosome analysis was performed by experienced cytogeneticists. Initially I hoped to carry out karyotype analysis with the help of an automated metaphase finder and karyotype analyser (Piper 1982) but this proved impractical.

2.4.1 Preparation, staining and analysis of chromosome spreads.

The chromosomes were prepared for analysis by high resolution 'G'-banding in the following manner developed by the MRC Human Genetics Unit. Three separate cultures were set up for each patient sample. 1ml of blood anticoagulated in Lithium-Heparin was added to the culture medium (8.3ml F10 culture medium, 1.5ml FCS, 0.1ml phytohaemagglutinin and 0.1ml 100X [GE]KST) in a glass universal container and incubated at 37°C for 72hrs. Two of the three cultures were blocked using 10^{-7}M methotrexate to synchronise the cell cycles and incubated for a further 16hrs. The third culture was arrested with 0.1ug/ml colcemid for 15mins and the cells harvested by centrifugation at 1200rpm (200g) for 8 minutes in a 15ml conical tube. The supernatant was removed, 8ml of 0.075M KCl was added and incubation continued at 37°C for 10mins to lyse the cells. Chromosomes were then fixed over 24 hours with 3 rounds of centrifugation at 200g for 8 minutes and addition of fresh fixative (3 parts absolute methanol 1 part glacial acetic acid).

The two cultures which had been blocked with methotrexate were released by replacement of fresh complete medium without methotrexate containing 10^{-5}M Thymidine in one and 100ug/ml BUdR in the other. After a further 5hrs 30mins incubation at 37°C, the cultures were arrested with colcemid and harvested as above.

Chromosome spreads were made by resuspending the fixed cell pellet in a small volume of fresh fixative and dropping on to a clean microscope slide. The unstained, air-dried spread was examined to assess the quality and spreading of metaphases and the cell density. When satisfactory metaphase spreads had been obtained the chromosomes were GT-banded (Giemsa-Trypsin). Slides were incubated for 1-3 hours in 2xSSC at 60°C, rinsed thoroughly in de-ionised water and incubated in 1% Difco Bacto Trypsin in PBS. After a further thorough rinse in de-ionised water, the slides were stained with 2.5% Giemsa (1.0ml Gurr's R66 to 38.0ml phosphate buffer pH6.8) for 30 minutes, rinsed briefly in de-ionised water, air dried, soaked in xylene and mounted with DPX.

Chromosome spreads were then analysed by the cytogenetic section of the MRC Human Genetics Unit.

2.5 DNA purification protocols.

The DNA purification protocols employed in this project follow well established phenol/chloroform extraction methods (Maniatis 1982) with slight modifications to suit the type and quantities of tissue involved.

2.5.1 Purification of DNA from fresh peripheral blood leukocytes.

10-20mls. of whole blood was mixed with an equal volume of lysis buffer (2% SDS, 0.1M Tris-HCl pH7.5, 20mM NaCl, 1mM EDTA) for 5 mins. An equal volume of water-saturated phenol was then added, mixed well but gently to minimise DNA shearing and the aqueous and phenol phases separated by centrifugation at 3000rpm for 10 mins. The aqueous phase was pipetted into a fresh tube and the DNA was precipitated by adding 1/2 vol. 7.5M NH4COOH and 2vols. isopropyl alcohol. Spooled DNA was then resuspended in 10mM Tris-HCl pH 7.5, 150mM NaCl, 10mM EDTA and treated with 50ug/ml RNase for 30 mins. at 37oC, followed by 100ug/ml proteinase K at 37oC overnight in the presence of 0.2% SDS. After sequential extractions using centrifugation at 3000rpm for 10 minutes with equal volumes of phenol, a 50:48:2 mix of phenol/chloroform/iso-amyl alcohol and an equal volume of 24:1 chloroform:iso-amyl alcohol, the DNA was precipitated from the aqueous phase by adding 1/2 volume of 7.5M NH4COOH and 2 volumes ethanol. The precipitated DNA was spooled, air-dried, dissolved in TE (10mM Tris, 0.5mM EDTA) stored in screw-top Eppendorf vials at 4oC until ready for analysis.

2.5.2 Purification of DNA from solid tumour material.

Tumour tissue was either processed immediately, fresh from the hospital pathology laboratory or stored in clean, sterile containers at -70°C. The fresh tumour tissue was frozen for 1-2 hours at -40°C to allow dicing of the sample. The tumour tissue which had been stored in the deep cold was diced while still solid. DNA was purified from approximately $1cm^3$ of tumour tissue and the remainder stored at -70°C. Dicing of the tumour samples was carried out by hand using a fresh single-sided razor blade for each tumour. The tumours were cut into cubes of less than 0.5mm and transferred immediately into lysis buffer described in section 2.5.1. From this point, the tumour samples were processed in the same manner as for whole blood. Tumour tissue tended to yield large quantities of DNA, requiring much larger volumes of TE for dissolution to give the preferred working concentration of 1mg/ml.

2.5.3 Purification of DNA from EBV transformed lymphoblastoid cell lines.

Lymphoblastoid cell lines were used as a renewable source of DNA for family studies. However, due to the practical problem of bulking up and feeding sufficient numbers of such cells from around 180 individual family members, cell line DNA was required only if there was a limited yield of DNA from fresh whole blood. This was the case in around 20% of samples. The DNA purification protocol for lymphoblastoid cell lines was essentially the same as that used for whole blood. $1x10^7$ cells were harvested by centrifugation from the culture medium and transferred immediately into the lysis buffer detailed in section 2.5.1. Once in lysis buffer, the lymphoblastoid cell line was dealt with in the same manner as for whole blood.

2.5.4 DNA purification from formalin-fixed paraffin-embedded archival material.

The method used here is derived from an amalgamation of published methods (Dubeau 1986, Goelz 1985, Warford 1988). Histologically confirmed normal tissue from deceased FAP family members was sectioned into 5um slices on the microtome to give around 100mg of tissue, including residual paraffin. Paraffin was dissolved in 10ml of xylene and removed with 3 xylene washes. The xylene was removed in 100% ethanol and the tissue progressively rehydrated in 99% ethanol (3 washes), 95% ethanol (2 washes), 70% ethanol (1 wash) and distilled water (1 wash). The tissue was then resuspended in 10mM Tris-HCl pH 7.5, 150mM NaCl, 10mM EDTA and treated with 100ug/ml proteinase K at 37°C overnight in the presence of 2% SDS. Phenol/chloroform extraction and ethanol precipitation was then carried out as for whole blood. DNA was recovered by centrifugation at 12000rpm for 30mins, resuspended in TE, treated with 50ug/ml RNase for 30 mins. at 37°C, followed by 100ug/ml proteinase K at 37°C overnight in the presence of 0.2% SDS. After phenol/chloroform extraction and ethanol precipitation, the DNA was pelletted by centrifugation at 12000rpm and redissolved in TE.

2.5.5 Estimation of DNA concentration.

DNA concentrations of genomic DNA and probe preparations were measured by optical densitometry on a Pye Unicam SP6-400 UV Spectrophotometer. Readings were taken at 260nm and 280nm and the 260/280 ratio of 1.8 taken as optimum purity of DNA. DNA was dissolved to a concentration of around 1mg/ml for ease of handling

2.6 DNA analysis with restriction fragment length polymorphisms

The methods described here are all well established (Maniatis 1989) and so an exhaustive list of references is not given. Key references for certain techniques are provided.

2.6.1 DNA digestion with restriction endonuclease.

5ug of purified DNA (1mg/ml) was used for each digestion. Electrophoretic rates were influenced by the volume of the test DNA required, due to differences in the amount of EDTA and salts in the digestion mix. Stock DNA samples were all kept at 1mg/ml and where necessary re-precipitated in 1/10 vol. 3M NaCOOH and 2.5 vols. of ethanol and redissolved in TE to 1mg/ml.

DNA samples were digested with the appropriate restriction endonuclease in a 40ul digestion mix in individually labelled, screw-top Eppendorf vials to avoid contamination from the water bath. The reagents were added in a set order to minimise the risk of contaminating one digestion mix with the DNA of another. Sterile distilled water was added first, the volume calculated depending on the required volume of test DNA solution. 4ul of 10X incubation buffer appropriate for the restriction endonuclease used for the digestion was then added (as provided by the manufacturers). Restriction endonuclease (Boehringer Mannheim, Northumbria Laboratories, Bethesda Research Laboratories or Biolabs) was then added at 4 units/ug of test DNA, usually requiring a volume of 2ul as the manufacturers supply the enzyme at a concentration of 8-12 units/ul. 5ug of test DNA was then added and the disposable pipette tip left in the Eppendorf tube until all DNA samples had been added. This minimised any possible risk of cross-contamination of DNA samples or a mix-up with the labelled tubes. The test DNA and the reagents were then gently pipetted up and down to ensure thorough mixing prior to the addition of spermidine, which might precipitate any localised concentration of the DNA. Spermidine was added from a stock solution of 100mM to give a concentration in the digestion mix of 5mM and the reagents gently mixed by pipetting. Spermidine enhances restriction endonuclease activity by mopping up inhibitory salts in the reaction mix.

The genomic DNA digestion mixtures were then incubated overnight in a water bath at the temperature appropriate for the enzyme in use. The majority of restriction enzymes required incubation at 37°C but a few required other temperatures for optimum activity (Taq 1 at 65°C, Bcl 1 at 50°C and BstX1 at 55°C). The sample tubes were spun down in the microcentrifuge the following morning and a further aliquot of 10 units of restriction enzyme was added to each

sample and incubated for a further 4 hours to ensure that the reaction went to completion. After complete digestion, the samples were ready for fractionation by gel electrophoresis.

2.6.2 Fractionation of digested DNA by gel electro-phoresis and Southern transfer.

Horizontal gel electrophoresis (Bethesda Research Laboratories electrophoresis tank) in agarose through TAE buffer (40mM Tris acetate 1mM EDTA) was used throughout for fractionation of the restriction endonuclease digested genomic DNA purified from blood leukocytes, cell lines or tumour tissue. The concentration of agarose in the gel depended on the size of the DNA fragments to be resolved, higher concentrations of agarose (eg. 1.5%) being used for resolution of smaller DNA fragments (eg. around 500bp) and 0.8% gels used for larger fragments (eg. around 10kb). The gels were run overnight with recirculation of the buffer through an ice-bath to avoid heating artifact. The voltage was adjusted between 1.5-4V/cm to give good separation over the 18hrs. of the run.

After digestion, samples were spun briefly in the microcentrifuge to ensure complete recovery of the DNA sample and the dye bromophenol blue with sucrose was mixed to a final concentration of 0.025% and 7% respectively. The dye allows more controlled manipulation of the sample and visualisation of the DNA sample in the well of the gel. It also serves as a marker when the electric field is applied to demonstrate how far the DNA fragments have run. The bromophenol blue runs with an electrophoretic mobility similar to 700bp of DNA. The sucrose serves as a carrier to ensure that the DNA sample sits in the well when loaded into the agarose gel.

Whenever a size marker was required, Hind III digested lambda phage was loaded into the first and/or the last well in the gel. This gives constant DNA fragments at 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 kb.

After electrophoresis, the DNA was stained with the fluorescent, intercalating dye ethidium bromide at 0.5ug/ml added to the running buffer for 30mins. The gel was then destained in distilled water for 10mins and photographed under ultra-violet light (260nm) to assess the quantity and quality of the DNA and the completeness of the digestion. The DNA was then rendered single stranded by denaturation in 1.5M NaCl, 0.5M NaOH for 45mins followed by neutralisation in 2M NaCl, 0.5M Tris HCl pH 5.5 for 60mins.

The fractionated genomic DNA was transferred to a nylon membrane by Southern blot (Southern 1975). A piece a 5mm Whatman blotting paper was spread over a glass plate suspended on a tank filled with 20X SSC so that the ends were immersed in the SSC and acted as a wick. The gel was placed on top of the 5mm paper and an appropriate sized piece of Hybond-N (Amersham International) was

laid on top of the gel. A glass rod was used to remove any air bubbles and two pieces of dry 3mm Whatman paper and a stack of dry paper towels were laid on top of the Nylon membrane. A rigid plate and a 1Kg weight were used to compress the paper towels to allow a flow of liquid from the reservoir through the gel and nylon membrane so that the DNA fragments were eluted out of the gel on to the membrane. The gel was surrounded with Cling-Film to prevent evaporation of the SSC. DNA transfer was complete overnight and then the filter air dried for 2-3 hours prior to permanent cross-linking of the DNA to the nylon by trans-illumination with ultraviolet light (302-305nm at 90W) for 3mins 30secs and baking at 80°C for 60mins in a vacuum oven.

2.6.3 Radiolabelling of DNA probes.

Radiolabelling of DNA probes was carried out by the random priming method (Feinberg and Vogelstein, 1983) using a commercial 'Multiprime Labelling Kit' (Amersham) according to the manufacturers instructions. 25-50ng of DNA probe was labelled either as plasmid or after endonuclease excision of the insert from the vector and purification of the insert. Probe DNA was dissolved in dH_2O in an Eppendorf centrifuge tube to give a final volume of 50ul and denatured by heating to 95-100°C for 2mins (insert) or 12mins (whole plasmid) with subsequent chilling on ice. 4ul of the unlabelled nucleotide solutions (dTTP, dATP, dGTP) provided with the kit were added on ice followed by 5ul of reaction buffer (Tris HCl pH 7.8, 50mM $MgCl_2$, 100mM 2-mercaptoethanol), 5ul of DNA oligo-nucleotide primer solution (aqueous random hexa-nucleotides with nuclease-free BSA), 50 uCi ^{32}P dCTP and 1-2 units of the 'Klenow' fragment of DNA polymerase 1 (in 50mM KPO_4 pH 6.5, 10mM 2-mercaptoethanol, 50% glycerol). After gentle mixing, the reaction mixture was incubated at 37°C for 30mins and the amount of incorporation of the ^{32}P labelled dCTP into the probe DNA measured by Cerenkov counting. 1ul of the reaction solution was transferred to a Whatman GF/C glass fibre filter disc. The disintegrations from the radio-isotope were measured in a scintillation counter as counts per minute (cpm). The disc was then washed with 5% TCA to precipitate the probe DNA and remove any unincorporated radiolabelled nucleotide. The scintillation count was repeated and the percentage and absolute incorporation of the radio-isotope calculated. Incorporation of more than 40% of the radio-isotope was accepted and labelling of the probe to a specific activity was in the range 0.5-$2x10^9$ cpm/ug of probe DNA.

Unincorporated radiolabelled nucleotide was removed from the radiolabelled probe solution to reduce non-specific background 'noise' signal during autoradiography. 200ug of sonicated salmon sperm DNA (to act as a carrier) and

1/10th vol (ie 7ul) of 3M NaCOOH, pH 5.2 with 2.5vols ethanol were added to precipitate the radio-labelled DNA probe. The solution was vortexed and the probe pelletted by centrifugation in a microcentrifuge at 12000rpm for 5mins. The supernatant was discarded, 500ul of sonicated salmon sperm DNA (10mg/ml) added to the pellet in the microcentrifuge tube and the solution heated to 95-100°C for 12mins to denature probe and salmon sperm DNA. The salmon sperm DNA blocks non-specific hybridisation of the probe DNA to sequences which are not uniquely human. The solution was then kept on ice prior to hybridisation to the genomic DNA digests on the nylon filter.

2.6.4 DNA hybridisation and autoradiography.

Prehybridisation and hybridisation of the digested genomic DNA on the nylon filters was carried out in double sealed polythene bags. The filters were prehybridised for 2-3hours in 25ml of the hybridisation solution (6X SSC, 5X Denhardt's solution: [1% Ficoll/1% polyvinylpyrrolidone/1% BSA], 0.5% SDS, 10% dextran sulphate) at 65°C, agitated in a water-bath. The radiolabelled DNA probe solution containing salmon sperm DNA was added to 2-3ml of hybridisation solution and mixed in a fresh 10ml tube to reduce the concentration of the radio-isotope at the point of insertion into the bag. This solution was then added to the hybridisation bag through a small hole and the bag resealed, avoiding entry of air bubbles. The hybridisation was carried out at 65°C overnight. For probes with a high content of repeat sequences such as MLZ 7.5 or MC5.61 (see description of probes), the stringency of the hybridisation was increased by carrying out the hybridisation at 69°C and with pre-annealing of the probe DNA to denatured sonicated total human DNA (5ug/ng of probe DNA).

After hybridisation, 2 x 5min low stringency washes (2X SSC, 0.1% SDS) were carried out followed by 2 x 5min high stringency washes (0.1X SSC, 0.1% SDS), all at 65°C The probed membrane was partially air dried and wrapped in cling film, DNA side up, with a 3mm Whatman paper backing prior to autoradiography.

The cling film wrapped membrane was then placed in direct contact with Kodak XAR film in a standard X-ray cassette for 24-48 hours at -70°C. The film was developed to reveal the hybridisation of the radiolabelled probe. If the signal was poor, the exposure was repeated and the X-ray film developed after 1-2 weeks.

2.7 DNA probes.

All DNA probes used in this project were kindly donated by workers who had cloned the markers as random DNA clones or were obtained through my collaboration with Dr Y Nakamura as part of work towards cloning the *APC* gene itself. The markers used in this project consist of DNA probe fragments which recognise natural polymorphisms at flanking restriction endonuclease cleavage sites and are known as restriction fragment length polymorphism (RFLP) markers.

2.7.1 Probe plasmid preparation.

All DNA marker probes were received as whole plasmid constructs and so there was no need for any ligation procedures to introduce insert into the vector. Plasmids were expanded to generate stock supplies in quantities sufficient for experimental work. The probe insert, incorporated into an appropriate plasmid vector, was introduced into E. Coli by standard techniques (Maniatis 1989), modified to the routine method used at the MRC Human Genetics Unit.

Stocks of E. Coli (strain JM83) rendered competent (receptive to the introduction of extraneous DNA) by a calcium chloride method (Maniatis 1989) are held at the MRC Human Genetics Unit. Around $1x10^8$ of these bacteria were transformed with approximately 25ng of whole plasmid by the following method. Plasmid solution was added to the bacteria, left on ice for 30 minutes, heat shocked at 42°C for 90 seconds and then put back in ice for 2 minutes. 800ul of L-broth with Mg^{2+} and 0.2% glucose were added and shaken at 37°C for 1 hour. A control of 10ng/ul of Bluescribe plasmid was treated in the same manner to assess the transformation efficiency. 50ul and 200ul of the culture medium was plated with a sterile glass spreader onto H Amp+Xgal plates (H agar, 50ug/ml ampicillin, 40ul of 20mg/ml Xgal per 20ml of agar) for pUC plasmid vectors and H Amp plates (without Xgal) for pBR322 vectors. The same volumes of 10ng/ul of the Bluescribe-transformed control bacteria were spread onto H Amp+Xgal plates. The plates were incubated upside down at 37°C overnight. Single colonies of bacteria which had undergone the characteristic blue-white colour change (implying both that transformation was successful and also that the transfected plasmid contained insert, since the expressed plasmid galactosidase gene is disrupted by the recombination of probe DNA insert into the polylinker cloning site of the pUC vector) were picked. These colonies were grown up in 500mls of T-Broth with 50mls of stock KH_2PO_4 at 37°C in the orbital incubator in order to provide bulk quantities of the cloned marker DNA fragment. All bacterial protein and DNA were removed and plasmid DNA purified by the following method.

The culture medium with the transformed bacteria was aliquoted into large tubes and carefully balanced before spinning at 6000rpm in a Sorval ultracentrifuge for 5 minutes to pellet the bacteria. The pellet was resuspended in 20mls GTE (1% of a 50mM solution of glucose, 25mM Tris pH8, 50mM EDTA) with 10mg/ml lysozyme and 40ml of alkaline SDS (1% SDS, 0.2M NaOH). The DNA in solution at this point comprised bacterial and probe DNA. The bulk of the bacterial DNA content was then removed by differential precipitation and centrifugation. 30mls of high salt (3M KCOOH pH 4.8 with acetic acid) was added and left on ice for 30 minutes, then spun at 12,000rpm for 30 minutes. The supernatant was strained through muslin and 0.6 volume of isopropanol added and left at room temperature for 5 minutes, before a further spin at 8,000rpm for 15 minutes. The resultant DNA pellet comprising plasmid and insert was then washed with 70% ethanol and air dried. The pellet was then dissolved in around 2mls of TE and the final purification steps involved ultra-centrifugation at 40,000rpm for 20 hours on a CsCl gradient (3.55g CsCl in 3.21ml + 0.34ml ethidium bromide 10mg/ml in a 5ml centrifuge tube). The plasmid band could then be visualised under UV light and removed by pushing a needle and syringe through the side of the plastic centrifuge tube at the level of the plasmid band and aspirating. The plasmid solution in CsCl was made up to 25ml in TE, taken up to 50ml with butan-1-ol and repeatedly extracted with butan-1-ol to remove the ethidium bromide. In order to remove CsCl from the plasmid preparation, the plasmid DNA was precipitated from the solution by adding 2 volumes of 70% ethanol at -70°C for 30 minutes then warming to 37°C and subsequently pelleting the DNA by spinning at 10,000rpm for 15 minutes. The pellet was redissolved in 1ml of TE and reprecipitated with 0.1vol. 3M NaOAc and 2.5 vols EtOH. The precipitated DNA was pelleted by centrifuge at 10,000 for 15 minutes. The DNA was taken up in TE, optical density measured to assess concentration and an aliquot run out on a mini agarose gel as a rough check of concentration and to assess the degree of RNA contamination. The preparations usually required removal of RNA by treatment with RNAase. A phenol chloroform extraction and salt reprecipitation was then carried out to remove excess RNAase enzyme.

The probe insert alone was used for PFGE studies and this was prepared by cutting the whole plasmid with the appropriate restriction enzyme to remove the insert from the polylinker plasmid cloning cartridge and fractionating on a mini agarose gel. Uncut probe, cut probe and a size marker (eg EcoR1/HindIII double-digested lambda phage DNA) were run out on an Ultra-pure Low Melting Point agarose gel, ethidium bromide stained at 5ug/ml and the probe insert band physically excised from the gel with a scalpel under UV light. The fragment of gel

with insert was then melted and stored in separate aliquots in Eppendorf vials in quantities suitable for a single radio-labelling by random priming as described in section 2.6.3.

The identity of each probe was confirmed by demonstration that the DNA probe insert was the appropriate size on a test mini gel according to details obtained from the worker who originally cloned the probe. The RFLP alleles generated were also checked to ensure that they were the appropriate sizes according to published probe data.

2.7.2 Description of DNA probes.

Details of insert size, locus recognised, cloning vector, restriction enzyme used to generate original cloned fragment (if different from vector cloning site), and original reference pertaining to each probe are shown in Table 2.1. C11P11 was originally from P. Scambler of St. Mary's Medical School, London. Probes pL5.62 and pEF5.44 were kindly donated by Y. Nakamura with whom I collaborated on the linkage mapping aspect of this project. These probes are subclones from a genomic cosmid library generated from a mouse-human hybrid with a portion of chromosome 5q as the only human component. Generation of the cosmid library, RFLP screening and sub-cloning into plasmid vector was carried out by Y. Nakamura and his group using a strategy similar to that previously reported (Nakamura 1988a).

Table 2.1 Probe identification details and original reference for each clone.

Probe (Human Gene Mapping No.)	Vector	Cloning site	Insert size (fragment)	Reference
		CHROMOSOME 5 PROBES		
λ MS8 (D5S43)	L47.1	BamH1	7.0kb	Wong 1987
MC5.61 (D5S84)	pUC18	Acc1	3.2kb (Taq1)	Nakamura 1988b
YN5.48 (D5S81)	pUC18	Acc1	2.4kb (Taq1)	Nakamura 1988b
EF5.44 (-)	pUC18	Acc1	2.3kb (Msp1)	New marker
L5.62 (-)	pUC18	BamH1	10.5kb (BglII)	New marker
ECB27 (D5S98)	Lambda phage	Sal1	2.8kb	Varesco 1989
C11P11 (D5S71)	pUC8	EcoR1	3.6kb	Bodmer 1987
pi227 (D5S37)	piAN7	HindIII/EcoR1	900bp	Stewart 1987
105-798Rb (D5S78)	pUC8	EcoR1	3.00kb	Leppert 1987
105-153Ra (D5S39)	pUC8	EcoR1	4.0kb	Leppert 1987
105-599Ha (D5S76)	pUC8	HindIII	4.90kb	Leppert 1987
L1.4 (D5S4)	pBR322	EcoR11	720bp	Pearson 1984
MLZ7.5 (-)	pUC18	EcoR1	2.5kb	Leppert 1987
		CHROMOSOME 17 PROBES		
YNZ22 (D17S30)	pBR322	BamH1	1.7kb	Nakamura 1988a
		SEX CHROMOSOME PROBES (for paternity testing)		
29C1 (DXYS14)	pUC9	Pst1	1.5kb	Cooke 1985

Probe and restriction enzyme combination recognising each polymorphic locus are shown in Table 2.2. The sizes of each possible DNA fragment generated for each polymorphic locus are shown. Published allele frequencies are shown for each RFLP and are documented in the relevant reference. However, as linkage analysis require knowledge of local allele frequencies, the results of a series of experiments to ascertain allele frequencies in a Scottish population are presented in Chapter 4 (Table 4.1).

Table 2.2 Polymorphism(s) recognised by each probe and references to original reports elucidating RFLP data.

Probe	Enzyme recognising polymorphism	Allele sizes (kb)	Reference frequency	
λ MS8	Multiple but Hinf1 used	Multiple (2.4-9.5)	90% heterozygosity	Wong 1987
MC5.61	Msp1	5.5/5.0	0.49/0.51	Nakamura 1988a
YN5.48	Msp1	9.0/8.0	0.51/0.49	Nakamura 1988a
EF5.44	Msp1	2.9/2.1	No published data	
L5.62	BglII	9.0/5.5	No published data	
ECB27	BglII	11.9/10.5	0.38/0.62	Varesco 1989
C11P11	Taq1	4.4/3.9	0.08/0.92	Bodmer 1987
			0.17/0.83	Leppert 1987
			0.11/0.89	Meera Khan 1988
pi227	Pst1	4.3/3.0	0.16/0.84	Stewart 1987
"	BclI	3.0/1.8/1.2	0.2/0.37/0.43	and
"	BstX1	2.7/2.3	0.3/0.7	Meera Khan 1988
"	MboI	0.55/0.45	0.44/0.56	
105-798Rb	Msp1	2.3/1.8	0.57/0.43	Leppert 1987
105-153Ra	Msp1	8.0/5.0	0.4/0.6	Leppert 1987
105-599Ha	Taq1	17.0/14.0/10.0	0.32/0.16/0.52	Leppert 1987
MLZ7.5	EcoR1	2.9/2.2	0.49/0.51	Leppert 1987
L1.4	EcoR1			Pearson 1984
YNZ22	Multiple, but Taq1 used	>10 alleles (1.3-10)	86% heterozygosity	Nakamura 1987
29C1	Multiple, but Taq1 used	>10 alleles (0.5-8.0)	hypervariable	Cooke 1985

2.8 Genetic linkage analysis.

The general concept of genetic linkage and linkage analysis are discussed briefly in this section. Further introductory explanations are given by Emery and Ott (Emery 1986, Ott 1986).

With regard to human diseases, genetic linkage analysis in its simplest form tests the degree to which a marker genetic trait (clinical, biochemical, cytogenetic or DNA polymorphism) and a disease are co-inherited, or exhibit genetic linkage. If there is complete non-linkage, such as might occur between a disease and a marker locus which are located on different chromosomes, then one might expect totally random inheritance of the disease and marker traits with no evidence of co-inheritance. There can also be incomplete co-inheritance of a disease and a marker trait, even when they have been shown to be close to each other using physical methods. This incomplete co-inheritance implies crossing-over (recombination) between the two loci. Such recombinations occur throughout the genome at the

time of meiosis and, although there are known to be recombination hot-spots, they are considered to occur randomly for the purposes of genetic linkage analysis. The proportion of such recombinant meioses out of all meioses studied is known as the recombination fraction and is a function of the genetic distance between the disease and the marker gene loci. The most widely used method of genetic linkage analysis is the maximum likelihood, or lod score, method. The odds ratio (expressed as $\log_{10}$ of the odds for linkage or log of the odds, acronym - lod score) that the observed data represent linked loci, as against chance, can be assessed by the equation $Z_{max}(\Theta)=\log_{10}[\Theta^{w}(1-\Theta)^{x}/(0.5)^{y}$ for phase-known, fully informative loci; $Z_{max}(\Theta)=$ peak lod -score achieved out of all recombination fractions (Θ) calculated; w = recombinant meioses, x = non-recombinant meioses and y = all meioses studied. The larger the lod score, the more faith one can have that the observed deviation from chance does indeed represent genetic linkage. The probability that loci are linked can be assessed for a given set of observations at all recombination fractions from 0.00 (completely linked) to 0.50 (unlinked). Generally, linkage is considered established when $Z_{max}(\Theta)$ achieves a lod score of 3.0 or greater. This corresponds to a ratio of the odds that the loci are linked to the odds on non-linkage of around 1:1000 chance at that recombination fraction. In some situations it is possible to use this equation to calculate lod scores by hand but because of confounding factors such as missing genotype data on some family members and reduced penetrance, most linkage analyses require a computer program as detailed below.

For a disease and a marker locus of unknown map locations, the recombination fraction at which the Z_{max} occurs provides a measure of the genetic distance between the two loci. There are mapping equations which calculate genetic distance (measured in centi-Morgans) from recombination fraction but in general, up to a recombination fraction (Z_{max}) of 25%, it is sufficiently accurate simply to convert the Z_{max} to centi-Morgans. Thus a peak lod score achieved at a recombination fraction of 5% corresponds to 5 centi-Morgans (or around 5 megabases of DNA). Clearly the more observations that are made for those two loci, the higher will be the peak lod-score, the narrower will be the 95% confidence (probability) limits and so the more certain will be the mapping data. Calculation of the 95% probability limits involves summing the area under the graph delineated by the lod score curve. 95% of this area is then calculated and applied to the graph, equally distributed about the peak lod-score and the 95% limits on the axis are then read off. An easier method, which approximates quite accurately to the 95% probability limits, is to reduce the peak lod-score by 1 unit and then read off the recombination fraction on the axis which corresponds to this lod-score above and below the recombination fraction at which the Z_{max} occurs. Thus genetic linkage

analysis allows the construction of a map of any given region based on the estimation of genetic distance between loci and also gives an assessment of the confidence with which that localisation fits the available data.

2.8.1 Recombinant mapping.

Genotypes for 6 probes (pi227, C11P11, ECB27, L5.62, EF5.44 and YN5.48; see Tables 2.1 and 2.2) were obtained and used to construct 7-locus haplotypes (including *APC*) for all family members. This facilitated the identification of recombinant events both between marker and disease loci and also in intervals between marker loci which also included recombinations between marker loci. Due to the number of markers studied, a number of recombinations were informative at multiple loci and so by analysing the grouping of these markers around the recombination event, it was possible to order some of the loci unequivocally into a recombinant map.

2.8.2 Linkage analysis.

Linkage analysis for the mapping study (presented in Chapter 4) was carried out using the MLINK package in the LINKAGE 4.7 group of programs (Lathrop 1985) on an Atari IBM-compatible desk-top microcomputer. No discrepancies in frequencies of recombinations between male and female have been observed (Leppert 1987, Meera-Khan 1988) and so the linkage analyses described here combine male and female data. The 1-lod unit of support was taken as representative of 95% probability limits. Multi-point linkage analysis was not felt to be valid for this study since only affected families were analysed, without CEPH (Centre Etude de Polymorphisme Humaine) data. Instead, the data are presented as a series of pair-wise analyses between marker and disease loci and between all pairs of marker loci. Linkage analyses for risk estimations were carried out towards the end of the project when a more powerful computer and a new version of LINKAGE were available. Risk estimations were calculated using multipoint linkage analysis with the MLINK program in LINKAGE 5.02 on an IBM Series 70 80386 computer with a math coprocessor.

Paternity testing was inherent in the multiple two-point analyses which were carried out as part of experimental work and any discrepancies would have revealed the possibility of non-paternity. In addition, all family members were genotyped for the hypervariable chromosome 17 probe YNZ22 (Nakamura 1987). All *APC*-marker obligate recombinants were also tested with the probe 29C1 which recognises a pseudoautosomal hypervariable sex chromosome polymorphism (Cooke 1985). Both of these probes are extremely useful for paternity testing since they have a high frequency of heterozygosity, producing multi-allelic systems and individuals can be

typed using standard Southern blot analysis. The probe 29C1 is extremely useful for paternity testing as Cooke found no two individuals out of 83 studied had the same genotype.

2.9 Allele loss analysis.

The polymorphic DNA fragments revealed by marker probes are derived from maternally and paternally inherited chromosomes. Loss of one of these alleles has been taken in the past to indicate loss of that whole chromosome, or at least a whole chromosome arm. However, intuitively this is incorrect and, as has been shown for chromosome 17p allele losses in colorectal cancer (Baker 1989), this assumption is not always, or even usually, a valid one. Allele loss simply implies an alteration at the probed locus and this can be due to deletion of a portion of DNA which includes the probed locus or whole chromosome loss. The remaining allele may or may not be reduplicated by non-disjunction or mitotic recombination. Reduplication of the remaining allele would be better termed acquired homozygosity. For the purposes of simplicity in this thesis, allele loss is considered a generic term encompassing all of these mechanisms. As discussed in Chapter 1, a high frequency of allele loss in tumour tissue with any given probe may imply inactivation of a tumour suppressor gene located close to the probed locus.

2.9.1 Assessment of allele loss at polymorphic loci.

The relative intensities of the bands revealed after hybridization of P^{32} labelled probe were compared in blood and/or normal colonic mucosa versus tumour tissue. Initially 2 controls were used comprising leukocyte DNA and normal colonic mucosa but when no allele losses were detected in colonic mucosa at any of the 13 loci tested, this policy was abandoned and only blood leukocyte DNA was used as control for the latter part of the study.

Loss of heterozygosity at probed loci was established when there was absence or marked diminution of one of the fragments revealed in the control tissue by probe DNA hybridisation. Initially an attempt was made to quantitate the intensity of alleles using laser densitometry. However, an empirical level of reduction in intensity had to be set which was no improvement on simply comparing allele intensities visually. The potential for mis-classifying the allele loss status of a tumour is due to the presence of small amounts of residual normal tissue and lymphocytes which reflect the host's constitutional heterozygosity. Only one published allele loss study has employed laser densitometry (Solomon 1987), all others have also relied on an element of subjective assessment of the relative allele

intensities. Accordingly, the results on allele loss assessment presented in Chapters 5 and 6 are based on visual assessment of allele loss status at each locus. In the vast majority of cases, recognition of allele loss was unambiguous but in those cases where there was some doubt, an independent assessment was obtained. If no agreement could be reached, then the tumour was classed as not informative at that locus. I am grateful to Dr. CM Steel for his assessment of these autorads.

2.9.2 Deletion mapping.

The central strategy of the analysis of colorectal tumours employed in this thesis is the construction of a high resolution genetic linkage map around the *APC* gene and then assessment of allele loss status at each of these loci and a further 6 previously mapped loci. This has allowed the delineation of the nature, extent and specificity for *APC* of any losses demonstrated. Allele losses can only be identified if the host is constitutionally heterozygous at the probed locus. Not all tumours are informative at every locus and hence precise mapping of the ends of deletions is not always possible. Therefore allele loss analysis indicates the maximum size of the deletion in any given cancer, although the lesion could be substantially smaller.

2.10 Pulse field gel electrophoresis (PFGE).

Digestion with restriction endonucleases which cut DNA only very infrequently due to the rarity of their recognition sites allows the generation of large DNA fragments. The fragments are so large as to require support to prevent non-specific shearing. This is achieved by performing cell lysis, DNA purification, restriction endonuclease digestion and loading of the high molecular weight DNA into the gel apparatus with the cells being analysed embedded in agarose throughout. Resolution of such large fragments of DNA requires a special gel electrophoresis apparatus which relies on a two dimensional electric field to separate fragments. The electric field is pulsed in one direction and then the other alternately to give the desired resolution depending on the duration of the pulse.

2.10.1 Embedding high molecular weight DNA in soft agarose plugs.

Cells used for pulse field gel electrophoresis were either EBV-transformed cell lines or lymphocytes separated from fresh blood by ficoll hypaque. In each case, cells were harvested and washed in phosphate buffered saline (PBS), counted and resuspended in PBS at 2.0×10^7 cells /ml of PBS. An equal volume of molten, 1% ultra-pure low melting point agarose in PBS was then added and aliquoted into a mould designed for the PFGE apparatus. The plugs set at 4°C and then pushed out

of the mould into 20 ml NDS(0.5M EDTA, 1% lauryl sarcosine, 10mM Tris pH 9.5). 10mg proteinase K was added to the NDS and plugs and incubated at 55°C for 48 hrs, with a complete change of solution and enzyme at 24hrs. The plugs were then stored for later use at 4°C in fresh NDS.

2.10.2 Digestion of DNA in plugs with restriction endonuclease.

It is important to remove all residual proteinase K from the agarose plugs to avoid digestion of the restriction endonuclease. Therefore, each plug was incubated at 50°C for 30 mins. in 5ml TE (10mM Tris, 0.5mM EDTA, pH7.8) with the powerful protease inhibitor, phenylmethyl sulfonyl fluoride (PMSF) (5ul of a freshly made solution at 20mg/ml in isopropanol). This was repeated with fresh TE and PMSF and then incubated at 50°C for a further 30 mins. The plugs were then washed twice in fresh 5ml TE at 4°C for 30mins and then twice at 4°C for 30mins in 1ml of the manufacturers restriction buffer for the appropriate restriction enzyme. 100ul of fresh restriction buffer, 5mM spermidine, 0.01% triton X and then 15 units of restriction endonuclease were added to the plug and incubated overnight.

2.10.3 PFGE and DNA transfer.

The digested DNA was size-fractionated by gel electrophoresis. Conventional unidirectional electrophoresis is not capable of resolution of DNA fragments over 30kb or so whereas 2 megabase fragments can be resolved by PFGE. The equipment used in this study was the CHEF-DR II (Bio-Rad Laboratories). Other equipment for PFGE consisted of the CHEF-DR II drive module, a Pulsewave 760 Switcher, a pump for circulating the buffer and a Model 200/2.0 Power Supply, all from Bio-Rad Laboratories. A Maxi-cool Varitemp liquid cooler (IMI Cornelius UK) was used to cool the circulated electrophoresis buffer.

Gels used for all experiments were 1% agarose gels in 0.5xTBE (Tris Borate EDTA buffer). The gel was cast in the mould provided with the electrophoresis equipment. The digested DNA samples in the plugs were then carefully transferred into the wells of the gel. A little molten agarose was then used to seal the plugs in the wells. Marker yeast chromosomes were added to at least one of the wells (Sacromyces cerevisiae, strain YP148). Gel electrophoresis was carried out in 0.5xTBE at 14°C and the conditions for each experiment are stated in the results section. In general the longer the pulse time, the larger the fragments resolved.

After electrophoresis, the gel was stained with ethidium bromide (0.5ug/ml of 0.5xTBE buffer) for 1 hour and then destained for 30 mins. in distilled water. After photography under ultra-violet light, the DNA in the gel was depurinated in 0.25M HCl for 30 mins., exposure to standard denaturing solution was for 50 mins. and

neutralising solution for 1 hour. The gel was then transferred to a nylon filter (Hybond-N, Amersham International) by standard Southern blot using 20xSSC, with a blotting time of at least 48 hours.

2.10.4 DNA hybridisation and autoradiography after PFGE.

Once immobilised on the nylon membrane, large DNA fragments separated by PFGE can be dealt with in the same manner as DNA fractionated by conventional electrophoresis. However, there are sequences in some of the marker yeast chromosomes homologous to PBR322 sequences which cross-hybridize to sequences in many plasmids. Such hybridisation produces very dense signals on autoradiography such as to interfere with the signals from the sequences homologous to the probe DNA. Therefore, probe insert alone, without any vector DNA sequences, was used in all PFGE hybridisation experiments. Autoradiography and development were carried out as described before in section 2.6.4.

2.11 Flow cytometry.

An attempt was made to analyse all sporadic colorectal cancers and adenomas by flow cytometry, in addition to a representative sample of 4 FAP adenomas. Although there was no problem with FAP adenomas, results from around 30% of the sporadic tumours were unsatisfactory. The reason for this was that there was a time period from the initial collection and DNA extraction until resampling for flow cytometry. There was evidence of tissue degradation over that time, thought to be due to a freezer thaw.

Flow cytometric analysis of the tumours collected for this project was carried entirely by Dr. CA Purdie of Edinburgh University Pathology Department. The method used was as follows: Portions of frozen tumour were thawed and prepared into suspensions of single nuclei by a detergent-trypsin method (Vindelov 1983). Chicken red blood cells served as internal controls, producing a peak equivalent to around 35% of normal human diploid DNA content. Nuclei were stained with 0.62M propidium iodide and analysed in a Coulter Epics CS flow cytometer at a wavelength of 488nm. Aneuploidy was considered present when two distinct G_0/G_1 peaks were visible and a DNA index calculated for each tumour (1.0 being diploid).

2.12 Pathology assessment.

Pathological assessment of all sporadic tumours was carried out at the Pathology Department of the Western General Hospital in the same manner as for

routine specimens. The sampling and macroscopic assessment of the fresh specimen was the only variation from routine practice in the department. As discussed in section 2.2.3, tumour samples for DNA analysis were taken adjacent to a block taken for histological assessment.

CHAPTER 3

CLINICAL SCREENING

3.1 Introduction

A number of family members from the 11 families which comprise the study population (KMD 1-6, 8, 10, 11, 14, 16) required clinical screening for FAP. Clinical screening of at-risk individuals was an important integral part of this thesis and ensured that the affectation status of all family members was both accurate and up to date. Confirmation of the accuracy of diagnosis became a major part of this work as a number of individuals had been reassured that they were not affected and discharged from follow-up but were actually affected. In two cases of such mis-classification, the gene defect had also been passed on to the next generation. The identification of a number of gene carriers who required prophylactic colectomy also facilitated the collection of adenomas from patients with FAP which tend to be encountered only rarely in routine surgical practice. An assessment has also been made of the frequency of extra-colonic stigmata of FAP in the study group. In addition to clinical screening, cytogenetic analysis was carried out on at least one affected member from each family in a search for a translocation which might be the key to cloning the *APC* gene itself and results of cytogenetic analysis are presented here.

3.2 Methods

At-risk family members were defined as the offspring of gene carriers. The age limit for classification as at-risk was 0-60 years and all such individuals were offered screening by rigid sigmoidoscopy as recommended by Bulow (Bulow 1987). Colonoscopy was carried out in a number of cases as was the routine practice of the consultant in charge of those patients. In all but a very few cases, I was personally involved in the clinical surveillance of the colon and rectum. Barium enema was also carried out in some patients at the request of the consultant in charge. All polyps were biopsied for histological confirmation of their adenomatous nature.

Two independent observers carried out retinal examination after mydriasis with 0.5% Tropicamide using direct and indirect ophthalmoscopy. Those with 3 or

more and/or bilateral CHRPE lesions were classified as CHRPE +ve as recommended by Chapman (Chapman 1989).

A number of affected family members were screened for the presence of mandibular osteomas by orthopantomography (OPG). The presence of an osteoma was confirmed by a radiologist who was unaware of the diagnosis. These lesion were defined as circumscribed homogeneously radio-opaque areas with no surrounding radio-lucent zone. All patients were examined clinically for such tumours and any with palpable lesions were invited for radiography. Children were not subjected to OPG as it would not have affected their clinical management and would therefore have meant subjecting them to unnecessary radiation.

Karyotypes were prepared and analysed from the peripheral blood lymphocytes of at least one affected member of each family.

3.3 Results

There were 38 previously diagnosed affected patients surviving in the 11 kindreds under study. The total number of affected individuals increases to 57 when cases are included who were designated as gene carriers on the basis of positive screening for polyps, CHRPE or osteomas carried out as part of this project. The overall numbers of at-risk family members screened and the new cases detected by each of the screening modalities are shown in Table 3.1. The number of new cases identified emphasises the importance of a clinician with a special interest in this disorder in avoiding mis-classification of gene carriers who might be discharged prematurely from screening. Registration of FAP families with a local clinical genetics department and, preferably, the setting up of a regional/national Polyposis register is vital.

Table 3.1 Overall numbers of at-risk family members screened and detection of gene carriers in the study population by each screening modality.

Total screened (any modality)	71
Number previously screened	15
New cases identified by:	
Any screening modality	19
Colonic examination showing adenomas	8
Retinal examination showing CHRPE	9
Presence of gross osteomas	2

3.3.1 Screening of the colon.

There were 50 individuals screened for colonic lesions including 5 patients who had undergone previous sub-total colectomy and ileo-rectal anastomosis who required surveillance of the retained rectum. One of those patients has subsequently required excision of the retained rectum due to excessive rectal polyp growth. Two probands (KMD 10/2-3, KMD 11/3-7) appeared to be new, sporadic cases of FAP and so 8 first degree relatives from KMD 10 and 12 from KMD 11 were

screened to ensure that the probands from these families (3-7 and 2-3 respectively) were indeed new mutations for FAP.

Ten patients were shown to have colorectal polyposis during the study period and the age range was 18-38 years. Two of these were patients who had been previously diagnosed as affected with only limited numbers of small polyps and had opted to undergo repeated colonic examination initially in order to postpone surgery. The remaining 8 cases were new diagnoses. In 4 cases the patients had previously been screened negative (KMD 2/3-4, KMD 2/3-6, KMD 8/4-4 and KMD 1/5-7; at ages 27, 23, 22 and 18 respectively), reassured that all was well and discharged from follow-up and in 3 of these cases the patients had offspring who also required screening. In the 4 remaining cases screening had never been carried out (current ages 18, 19, 23 and 25).

Eight of the ten patients diagnosed during the study period have now undergone prophylactic colectomy (1 proctocolectomy at the patient's request, 1 total colectomy and ileal pouch and 6 sub-total colectomy with ileorectal anastomosis). Adenomas harvested from 4 of these cases were used for the assessment of allele losses as presented in Chapter 6. The remainder have been stored for further analysis when the *APC* gene itself is cloned. Polyps detected during this screening program were small sessile lesions with the largest encountered only 1.5cm in diameter. The polyp from this patient (KMD 1/5-7) was in the caecum and showed very dysplastic appearances amounting to carcinoma *in situ* (see Figure 3.1d). The typical macroscopic appearances of polyps detected in this patient population is shown in Figure 3.1 (from patient KMD 1/5-17). Some of the adenomas were extremely small and amounted to monocryptal neoplastic changes (Figure 3.1c)).

Fig. 3.1 a. Macroscopic appearance of the mucosal surface of the sigmoid colon of a colectomy specimen from patient KMD 1/5-17 showing small, flat, sessile polyps typical of the FAP adenomas occurring in this patient population.

b. Histological features of one of the polyps from patient KMD 1/5-17.

c. Monocryptal adenoma from patient KMD2/2-6.

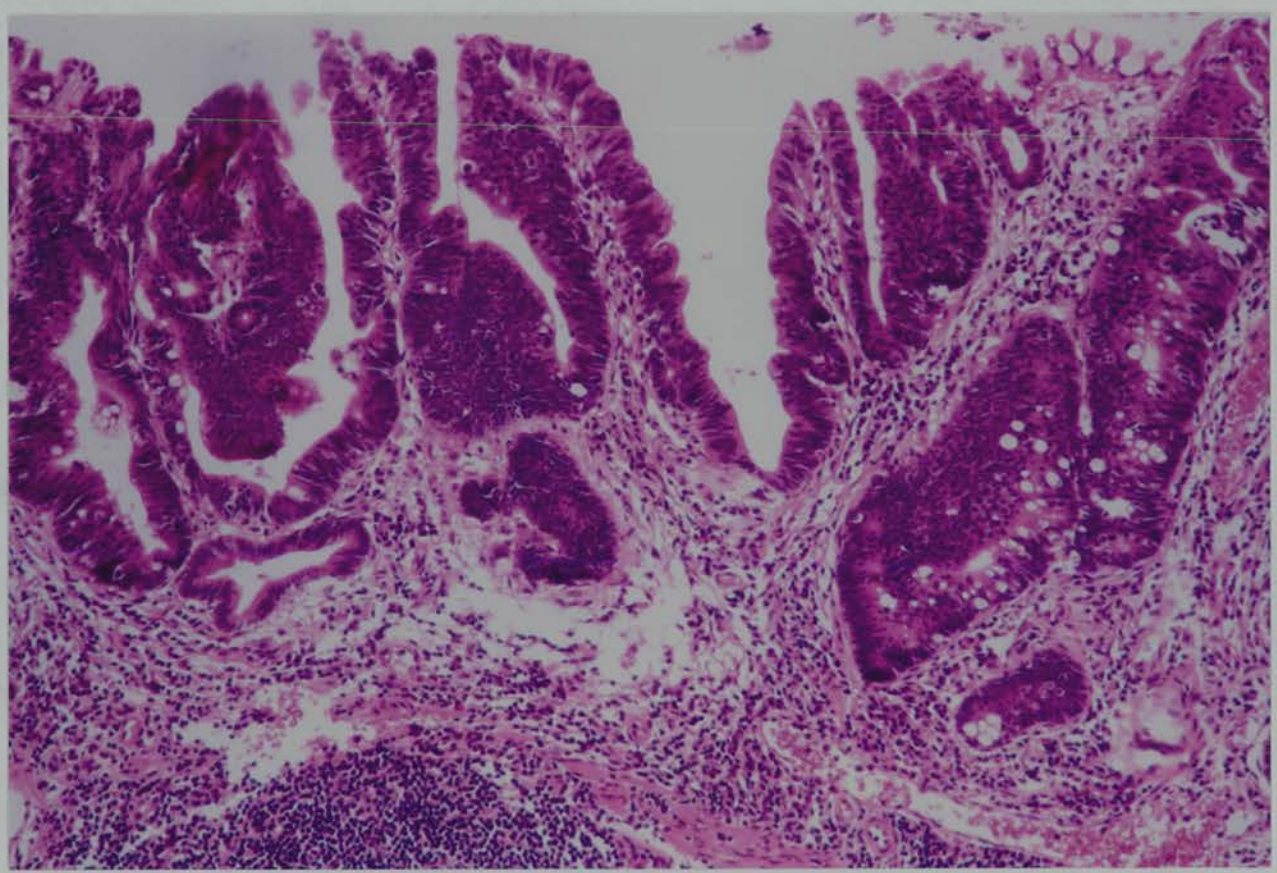
d. Histological features of a 1.5cm flat caecal adenoma from individual KMD 1/5-7 showing severe dysplasia amounting to carcinoma *in situ*.

a.

b.

c.

d.



Thirty patients were screened negative for the presence of colonic polyps (age range 14-64). This total includes the parents and siblings of the sporadic cases of FAP (KMD 11/3-7 and KMD 10/2-3), who can all be reassured that they are not affected and that they cannot have passed on the defective gene. The remaining family members have all been assigned residual risk estimations as detailed in Chapter 8 and the requirement for further follow-up will henceforth be tailored to that risk. The continued commitment to those individuals still requiring screening has been passed on to a local surgeon with an interest in colorectal disease or back to the original consultant in charge.

3.3.2 Retinal screening

Retinal examinations were carried out on a total of 54 family members. The numbers of individuals screened and the findings for each group classified by disease status is shown in Table 3.2. Seventy-four percent of the affected individuals had eye lesions and a further 2 affected individuals had less than 3 CHRPE lesions and hence are classified as CHRPE -ve. Five of the CHRPE -ve gene carriers came from one family (KMD 8) in which the CHRPE phenotype only affects one *APC* gene carrier. The remaining CHRPE -ve gene carrier was a sporadic case of FAP (KMD 10/2-1). None of her children have eye lesions and they are too young for colonic screening.

Table 3.2 Retinal screening for FAP in 54 family members comprising gene carriers (as shown by the presence of colonic polyps), non-gene carriers and adults who had never been screened or children under the age for colonic screening (uncertain phenotype).

Disease status	Total	CHRPE	
		+ve	-ve
Gene carriers	23	17(74%)	6(26%)
Non-gene carriers	13	0(0%)	13(100%)
Uncertain phenotype	18	9(50%)	9(50%)
Total screened	54	26(48%)	28(52%)

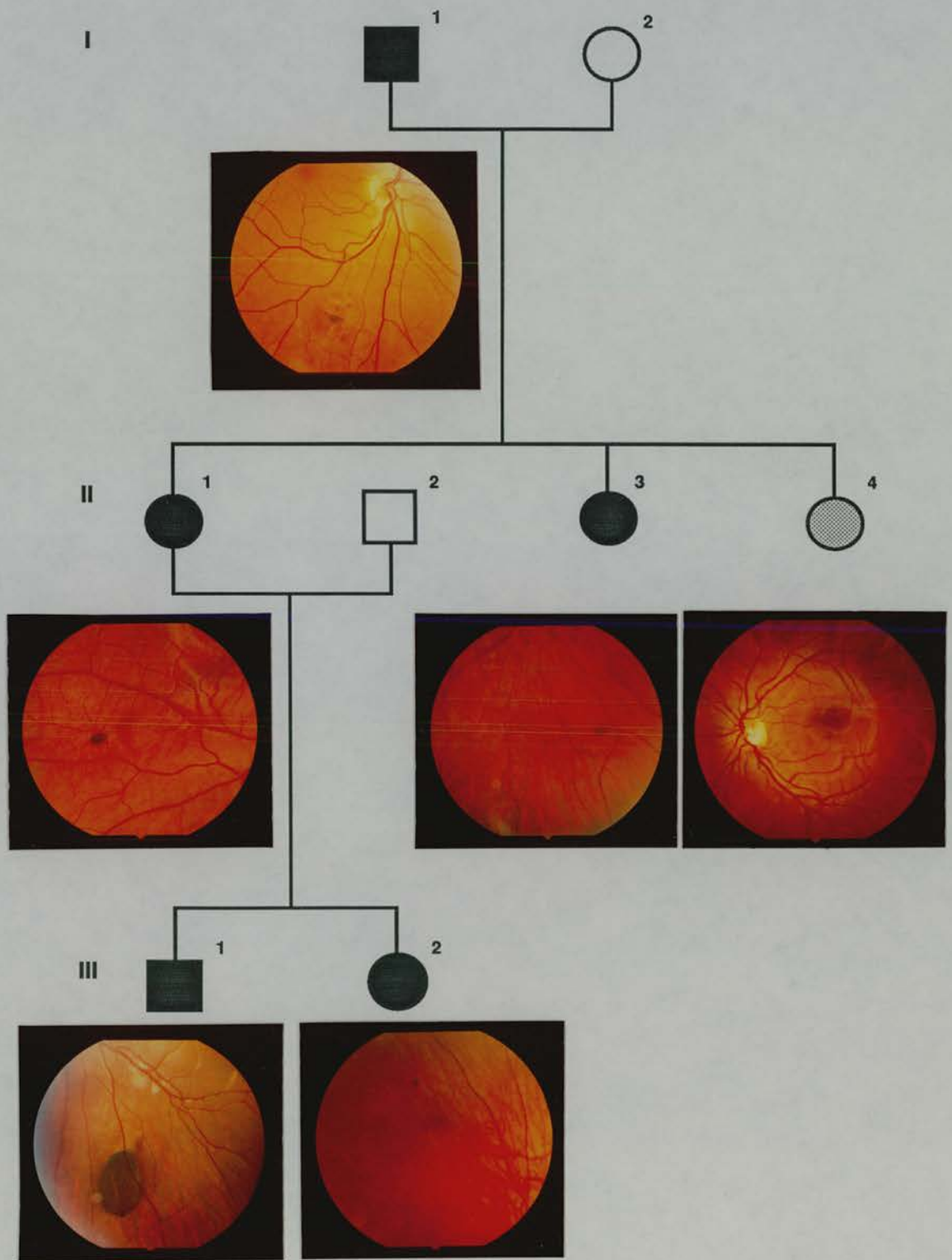
The youngest child examined was 3 years of age and an excellent view was obtained with no CHRPE lesions present. The youngest child shown to have CHRPE lesions was 4 years old and so the lesions certainly appear to arise prior to the development of polyps and may well be congenital. Eight children and one adult who has refused colonic examination or treatment were diagnosed as being affected solely on the presence of typical CHRPE lesions. There were 2 cases where the eyes lesions were present in unaffected individual but these were a solitary unilateral

lesion in one case and 2 lesions in one eye in another. There were 13 individuals who were negative for both retinal and colorectal lesions. Overall the data presented here suggest that if one affected family member exhibits CHRPE lesions then for the rest of that family colonic polyps and eye lesions appear to be co-inherited as part of the FAP phenotype. The presence of CHRPE lesions in a child under the age for colonic screening indicates the inheritance of the mutant *APC* allele. Although the overall penetrance of CHRPE is 74%, for families with CHRPE as part of the FAP phenotype the penetrance is 100%.

Typical examples of the various appearances of CHRPE lesions are shown for family KMD 3 in Figure 3.2 and demonstrate the co-inheritance of retinal and colonic stigmata of FAP. Patient KMD 3/1-1 shows an area of pigmentation and depigmentation, KMD 3/2-1 shows a densely pigmented lesion, KMD 3/2-3 shows a pigmented lesion with a halo of depigmentation (not well seen in the print) and KMD 3/3-1 had several of these large (up to 3 disc diameters) pigmented lesions. A normal fundal photograph from unaffected individual KMD 3/2-4 is shown for comparison.

Figure 3.2

Pedigree KMD 3 showing co-inheritance of colonic polyps and CHRPE lesions in two generations. Both children in the youngest generation have inherited the gene mutation since they have CHRPE lesions, although they are below the age where colonic screening is required.



3.3.3 Screening for osteomas and epidermoid cysts.

A total of 15 patients with FAP were assessed for the presence of cranio-facial osteomas. Nine patients (60%) had one or more lesions of the cranium or the mandible. Four patients had lesions in the mandible with an average of 2.5 tumours (range 1-6). In two cases the tumours were symptomatic, with pain and tenderness at the angle of the mandible. Figure 3.3 shows an example of large symptomatic bilateral mandibular osteomas (from patient KMD 11/3-7).

Figure 3.3 Example of 1cm bilateral osteomas at the angle of the mandible (from patient KMD 11/3-11).

Osteomas of the cranial vault occurred in 5 cases. No patients had both mandibular and cranial osteomas. However, it is notable that cranial and mandibular osteomas affected different individuals within the same family and so the particular gene mutation causing the disease in any given family does not appear to have a direct influence on the site of osteoma formation. Two at-risk children (KMD 8/4-5 and 4-6) under the age requiring colonic screening had such gross cranial osteomas as to allow a confident clinical diagnosis of FAP before puberty and this is confirmed on DNA analysis as shown in Chapter 8.

In addition to the presence of osteomas, an assessment was made for the presence of other extra-colonic features of FAP. Of the 46 surviving patients designated as obligate gene carriers on the basis of having colonic adenomas, only one patient (2%) had desmoid disease out of the 11 families studied. While it is difficult to test for the presence of desmoid disease, it is unlikely that any of these lesions would be missed since the diagnosis is usually made early in the course of tumour growth (Bulow 1987).

I was able to carry out a thorough examination of the skin to determine the presence or absence of epidermoid cysts in 22 affected family members and these lesions were present or had been previously removed from 6 patients (22%). They were mostly multiple about the head and face but frequently had occurred as solitary lesions before puberty.

3.3.4 Cytogenetic analysis.

The results of cytogenetic analysis were disappointing but predictable since karyotypic abnormalities associated with FAP have been reported in only 3 patients from two families in the world literature to date (Herrera 1986, Hockey 1989). In this study, there were no karyotypic abnormalities which could be substantiated. One karyotype from a sporadic case appeared to show a small interstitial deletion in the region 5q21-22 which was present in more than 40% of cells examined (Figure 3.4). However this apparent small deletion was due to artifact since the patient was re-sampled and a new spread carried out which showed a completely normal karyotype (Figure 3.5). Cytogenetics was an integral part of this project but unfortunately has not provided useful data.

Figure 3.4 Provisional karyotype from an affected individual in whom the FAP syndrome arises as a result of a new *APC* mutation (KMD 10/2-3). The arrow indicates what initially appeared to be an interstitial deletion but which was not confirmed by repeat analysis.

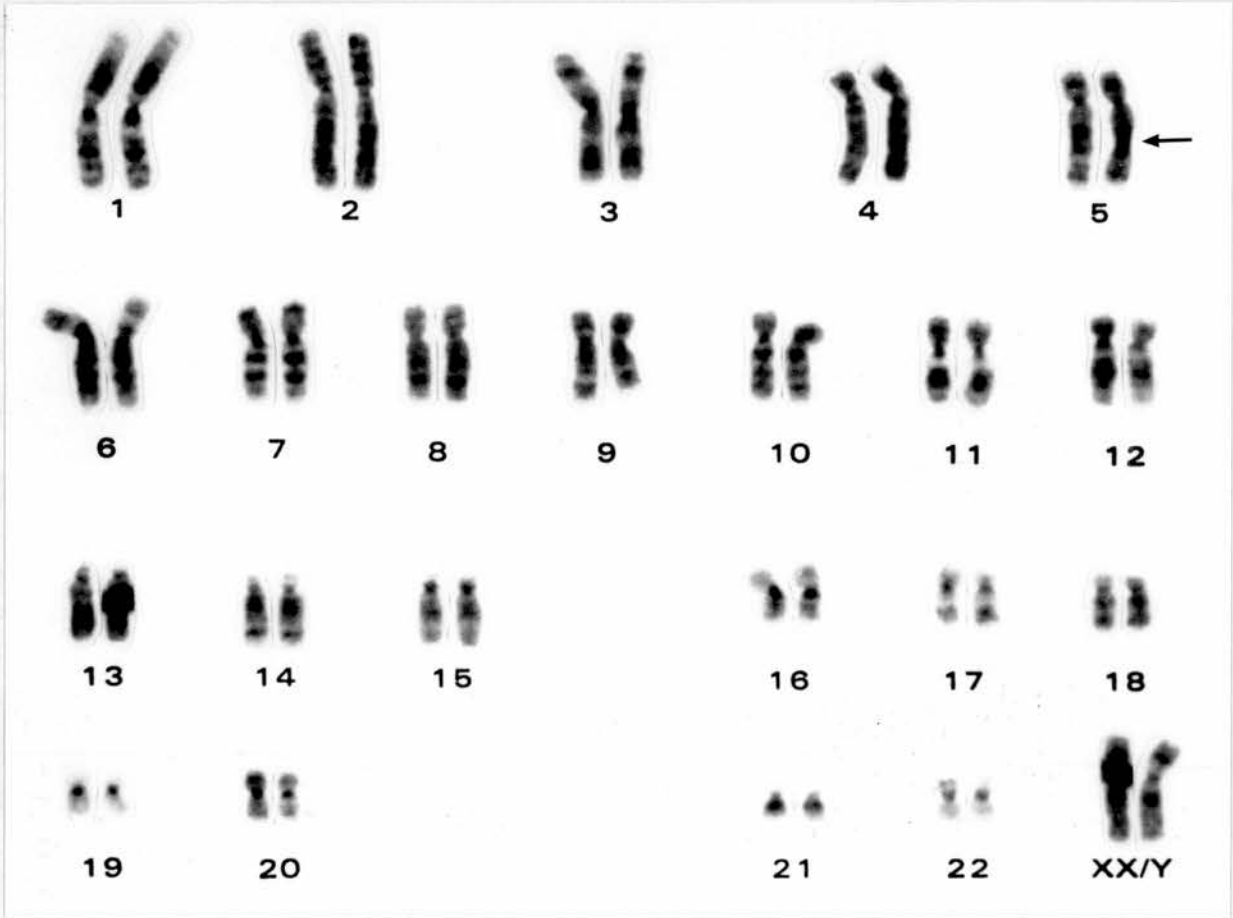
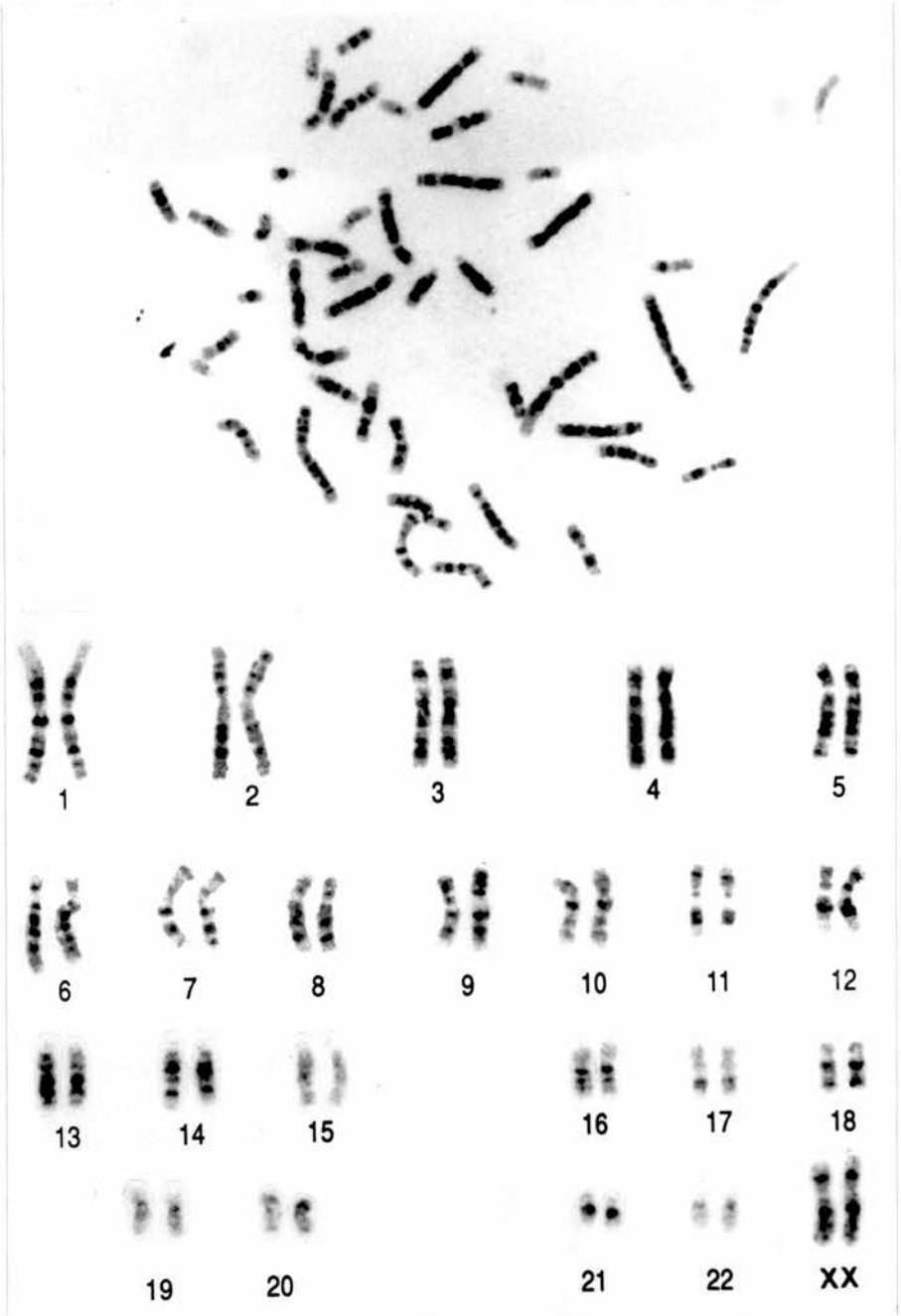


Figure 3.5 Karyotype of the individual shown in Figure 3.4 after re-sampling and re-preparation of the chromosome spread, showing two completely normal chromosome 5 homologues in all cells analysed.



3.4 Discussion

The success of this project should not only be measured in terms of academic advances in the understanding of FAP and colorectal cancer, but also in terms of the number of patients who have been identified as gene carriers and have now undergone successful prophylactic treatment, but who would never have been screened otherwise. A number of these patients had been unjustifiably reassured that they were unaffected and one of these patients had carcinoma *in situ* in the resected specimen. There can be little doubt that a number of these patients would have developed an invasive carcinoma without appropriate treatment. The reason for this success in prophylaxis is solely due to a particular interest on the part of a clinician and in general, the routine care of these families prior to this project has been less than optimal. The results of the screening program presented here only serve to strengthen the proposal that follow-up of patients at risk of FAP should be formalised into a regional or centralised register (Gunn 1990, Macrae 1990, Vasen 1990). Such a register would be entrusted with ensuring that screening was not only of a high standard but also was not discontinued prematurely. In addition, it should also ensure that timely prophylactic surgery is undertaken.

The method of surveillance of at-risk patients deserves some comment. Initially I followed the recommendations of Bulow (Bulow 1987) that only rigid sigmoidoscopy was required as all affected individuals eventually developed polyps in the rectosigmoid. I now feel that formal bowel preparation as an outpatient with a laxative such as sodium picosulphate and examination of the rectum, sigmoid and descending colon with a fibreoptic sigmoidoscope is preferable. The reason for this change is the certainty of affectation status afforded by DNA analysis which is detailed in Chapter 8. If DNA analysis predicts that the patient is affected with more than 99% certainty but polyps are not present in the rectum then the clinician should be able to visualise the colon well proximal of the rectum to ensure that it is not a case with relative rectal sparing. The use of the fibreoptic scope also allows multiple random biopsies to be taken in cases where the adenomas are still very small flat lesions. In all 8 cases where colectomy was carried out the diagnosis would have been confirmed since polyps were present in the sigmoid despite relative rectal sparing in 2 cases.

The demonstration of pigmented eye lesions in a 4 year old child in this study and previously in a 3 month old child (Traboulsi 1987) confirm the congenital nature of these lesions. The overall sensitivity of CHRPE lesions in indicating the presence of colorectal polyps was 74% but in those families which had retinal lesions as part of the syndrome, CHRPE lesions faithfully co-segregated with

colorectal polyps in 100% of cases. Hence it is important to examine the affected parent to assess the family category before assigning affectation status to an at-risk individual, particularly in the case of children below the age requiring colonic examination. The nature of the lesions detected here are similar to those described elsewhere (Iwama 1990, Traboulsi 1987) and the numbers and laterality of the lesions were also comparable (Chapman 1989, Iwama 1990, Polkinghorne 1990, Traboulsi 1987). Chapman reported that there were no exceptions to the presence of CHRPE lesions in gene carriers in 25 different families from Northeast England (Chapman 1989) but this is at odds with the findings of this study and of others (Iwama 1990, Traboulsi 1987) that certain families do not have eye lesions. However, since publication, the Newcastle group has found more than one family which do not have CHRPE lesions (J Burn personal communication 1990). The CHRPE phenotype provides a useful once-and-for-all screening tool which is independent of colonic screening which has the disadvantage of age-dependent penetrance. The two screening modalities can be combined with RFLP linkage data using Bayesian methods to allow the assignment of a combined risk which can then influence subsequent patient management and this work is presented in Chapter 8.

In this series 60% of patients with FAP had overt or occult cranio-facial osteomas which is lower than in other reported series (Bulow 1987, Jarvinen 1982, Ushio 1976, Utsunomiya 1975). This discrepancy is probably due to the smaller numbers studied here but may be due to differences in the patient population studied. Differences in anatomical distribution of osteomas in different members of the same family and the presence in some family members and not in their affected relatives suggests that genetic factors are not the only determinant of the FAP phenotype and that other factors must play a role in the development of these lesions. It is difficult to postulate what these factors might be but perhaps the genetic lesion results in an abnormal response to minor bony trauma in the affected areas. Hence only patients who traumatised the mandible or skull at a certain age or point in bone growth would develop osteomas.

Desmoid tumours occurred in 2% of this study population which fits well with other reported series (Bulow 1987). The patient with an intra-abdominal desmoid tumour was extremely unfortunate, having undergone prophylactic colectomy with an early carcinoma in the resected specimen, only to develop a painful untreatable benign tumour which has caused her a great deal of distress. The affected patient was a 39 year old female which is the typical sex and age group (Bulow 1986).

Epidermoid cysts were detected in 22% of this study group which agrees reasonably well with published series which range from 3% (Bulow 1986) to 53%

(Leppard 1974). In this series, the lesions had developed in childhood in 3 of the 6 patients (50%) with epidermoid cysts. It is important that clinicians take note of the presence of multiple epidermoid cysts as such lesions are extremely uncommon in normal individuals before puberty (Leppard 1974) and so may indicate the development of FAP at a later date.

The personal undertaking of clinical screening of colorectum and retina of these patients has been an important aspect of this project. The validity of affectation status has been assured in every case and the fundamental clinical basis of the linkage analysis is sound (presented in Chapter 4). The personal relationship which I have had with these families has given me great insight into their problems and what they would hope future developments in treatment and blood tests using DNA analysis might be able to offer them.

CHAPTER 4

GENETIC LINKAGE ANALYSIS

4.1 Introduction

This chapter describes the construction of a genetic linkage map of 6 marker DNA probes recognising restriction fragment length polymorphisms around *APC*. Genotypes were obtained for all 6 probes in every member of the kindreds described in Chapter 2 in whom a blood sample or archival pathology material was available. The data presented here comprise identification of crucial crossings-over which have allowed recombinant mapping and confident ordering of parts of the map. The data are also presented as a series of two-point linkage analyses between each marker and *APC*, and also between each marker and all other markers.

4.2 Methods

Kindreds KMD 1-6,8,14 and 16 comprise the nine families which were selected for study on the basis of informativity, with sufficient meioses to provide data for genetic linkage analysis (see Appendix A). 155 individuals have been genotyped and phenotyped. This provided in excess of 90 meioses for linkage analysis if all families were fully informative for any marker.

Clinical, pathological and genealogical ascertainment of all kindreds is described in Chapter 2. The data presented in this chapter and in Chapter 8 overlap to a certain extent in that this final mapping exercise includes a number of individuals who were assigned risk estimations and subsequently screened clinically as part of this project. In chapter 8 the recombination fraction for linkage of each probe to *APC* was derived from an amalgamation of published data, supported by order derived from the deletion analysis shown in Chapter 5 and including data from a preliminary linkage analysis not including those assigned risk estimations. I have not shown the results of this analysis for the sake of clarity but there were no substantial differences from those shown here in the final analysis, only the lod scores were somewhat lower.

For the purposes of this genetic linkage analysis, penetrance of FAP was assigned as 1.0 for family members in whom colorectal screening had been personally undertaken and who were over the age of 30 years. Individuals over 15

years of age who did not have CHRPE lesions but are members of families which have been demonstrated to exhibit CHRPE as part of the FAP phenotype (KMD 1,2,3,6) and were screened negative for polyps were classified unaffected. If an individual was negative on colonic examination but not part of a CHRPE expressing family, then that individual was assigned to the 'FAP unknown' category. Affectation status of those under the age of 15 years was ascertained by fundoscopy alone as such children were not subjected to colorectal screening. The status of 8 affected and 5 unaffected children who were members of families which expressed the CHRPE phenotype were ascertained by this method (see Chapter 3).

Genotyping was carried out as described in Chapter 2.5-2.8. DNA was purified from lymphoblastoid cell lines wherever possible to conserve DNA stocks but in a number of cases, DNA was extracted from fresh peripheral blood leukocytes as cell lines from these individuals were not available. Genotypes were obtained for 6 DNA probes which recognise restriction fragment length polymorphisms on chromosome 5. DNA purified from 50 unselected individuals, comprising leukocyte DNA from laboratory staff, or DNA purified from fresh placenta was also genotyped, in order to ascertain allele frequencies for all marker DNA probes in a control Scottish population.

Recombinant mapping using rare recombinant events informative at 3 or more loci have allowed confident locus ordering for some parts of the map. Linkage analysis was carried out as described in Chapter 2.8.2. The lod scores for linkage between markers are relatively low because the families were not specifically selected for a high level of informativity such as is the case with CEPH families. As described in Chapter 2, birth certificates were checked for all family members as part of the process of kindred ascertainment. Precautions to ensure valid paternity by molecular genetic techniques are also described in 2.8.2.

4.3 Results

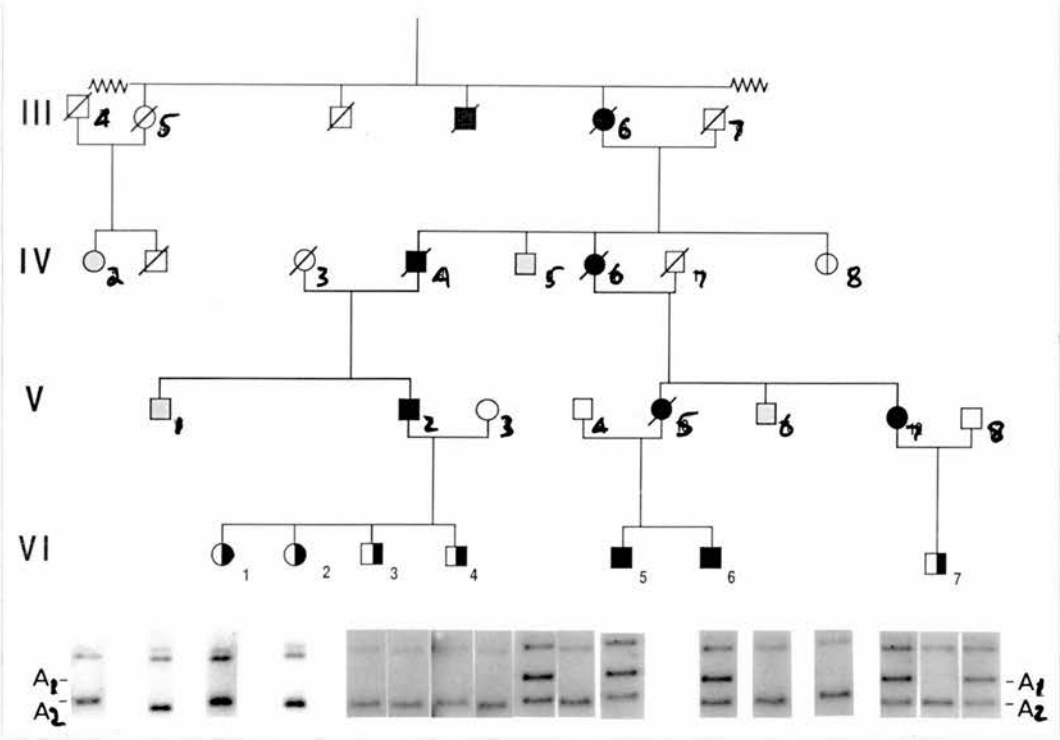
The results of the experiments to ascertain the allele sizes and frequencies in a local control population are tabulated in Table 4.1. These data were then incorporated into the LINKAGE computer programs during the linkage analyses.

Table 4.1 Allele size and frequency recognised by 6 polymorphic chromosome 5q DNA marker loci in a control Scottish population. The restriction endonuclease allowing recognition of the polymorphism at each probe locus is given in brackets.

Probe (Enzyme recognising polymorphism)	Freq./size (kb) A1	A2	A3	Number of chromosomes examined
EF5.44(Msp1)	0.18/2.9	0.82/2.1		84
L5.62(BglII)	0.93/9.0	0.07/5.5		76
YN5.48(Msp1)	0.45/9.0	0.55/8.0		60
ECB27(BglII)	0.39/11.9	0.61/10.5		74
C11P11(Taq1)	0.14/4.4	0.86/3.9		100
pi227(Pst1)	0.25/4.3	0.75/3.0		66
pi227(Bcl1)	0.17/3.0	0.46/1.8	0.37/1.2	54
pi227(BstX1)	0.29/2.7	0.71/2.3		28
pi227(Mbo1)	0.25/0.55	0.75/0.45		20

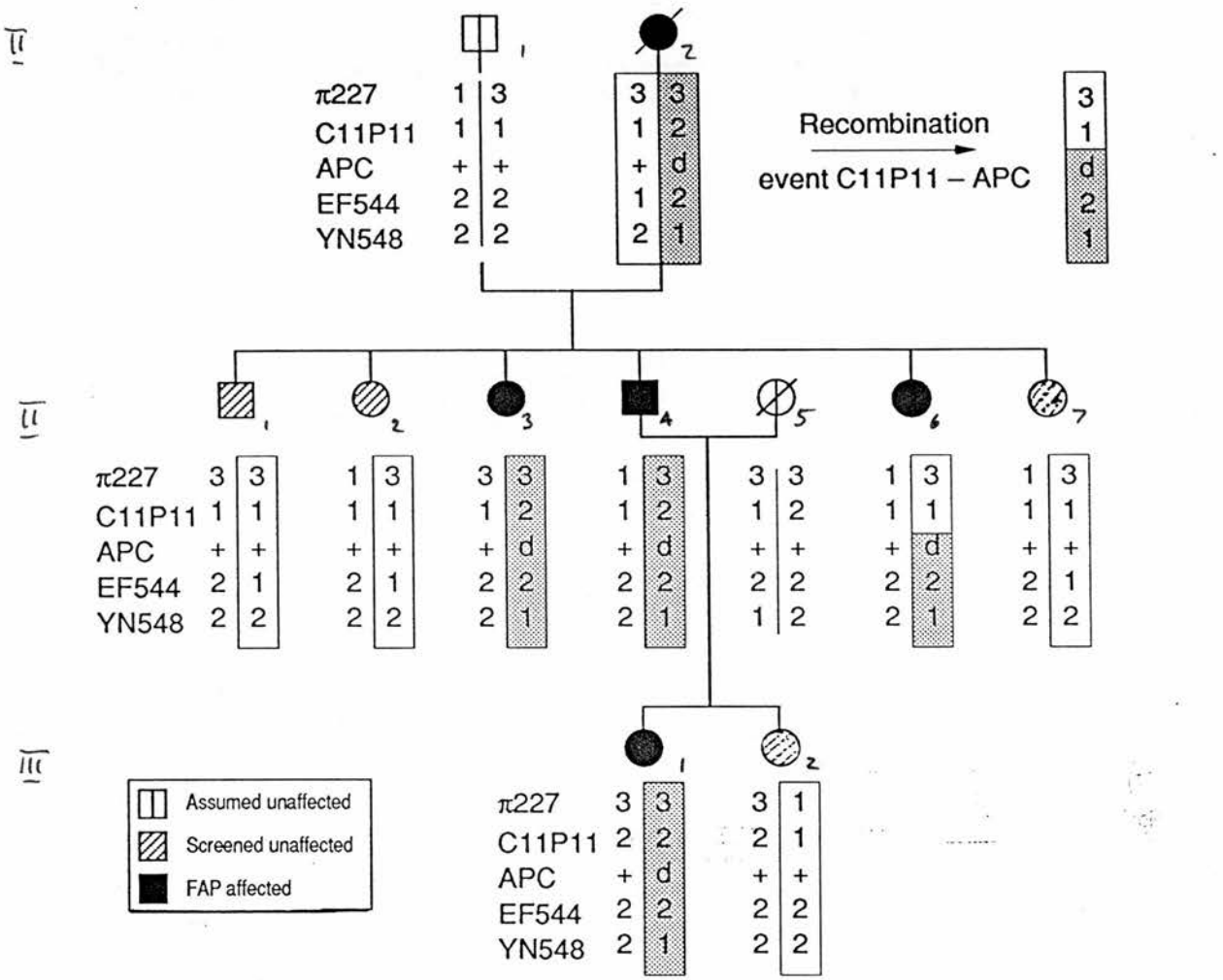
Complete raw data on affectation status and genotype information for all probes studied are shown in Appendix B and can be correlated directly with the respective pedigrees in Appendix A. An example analysis of C11P11 genotype for individuals in part of KMD 1 is shown in Figure 4.1. The disease segregates with the (rare) A1 allele in this large family and so 6-7 or his father is recombinant for C11P11-*APC*.

Figure 4.1 Part of KMD 1 pedigree showing C11P11 alleles in three generations.



Twelve critical recombination events were identified where markers were informative at 3 or more loci thereby allowing ordering of loci in 5q21-22. An example recombination event is shown in Figure 4.2 where a crossing-over has occurred in the interval between C11P11 and *APC* which is cancelled by both YN5.48 and EF5.44. The small units of genetic distance between loci (see below) preclude any double recombination events and so YN5.48 and EF5.44 must be on the *APC* side of C11P11 and are most likely on the opposite side of *APC* from C11P11 given the recombination fractions (Tables 4.3, 4.4). The phenotype data shown in Figure 4.2 are those after the final screening round. Figure 4.2 is also shown as Figure 8.1 prior to the final round of screening.

Figure 4.2 Example family (KMD 2) showing FAP disease status and genotypes for markers pi227, C11P11, EF5.44 and YN5.48. The chromosome carrying the disease (d) gene is shaded and there has been a recombination between C11P11 and *APC*.

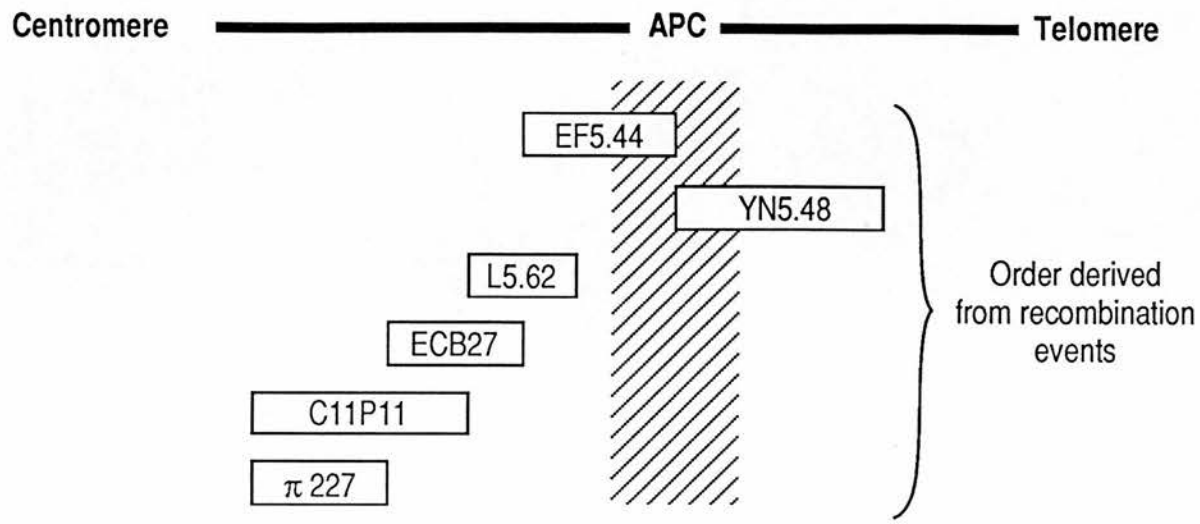


The results of identification of other important rare recombinations in these families is detailed in Table 4.2 with each of these recombination events grouping loci together in different sets. All recombination events which were fully informative for 3 or more loci are shown with the vertical line representing the recombination event in each case and the informative markers ranged either side of the breakpoint depending on the haplotypes resulting from the recombination. This allows the construction of a composite recombinant map of loci as shown in Figure 4.3. The shaded area represents all possible locations of *APC* relative to the markers studied. Thus, *APC* could overlap EF5.44 or YN5.48 but cannot overlap L5.62, ECB27, C11P11 or pi227 due to the presence of recombinants.

Table 4.2 Recombination events where 3 or more loci were informative. The vertical line represents where the recombination has occurred in relation to each of the informative loci.

C11P11 pi227	*APC* EF5.44 L5.62	C11P11	*APC* EF5.44 YN5.48
L5.62 pi227	*APC*	pi227	*APC* EF5.44 L5.62
ECB27 pi227	*APC*	L5.62 C11P11 pi227	YN5.48
ECB27 C11P11	YN5.48	ECB27	EF5.44 YN5.48
ECB27 pi227	YN5.48	ECB27 C11P11 pi227	EF5.44 YN5.48
EF5.44 ECB27 C11P11 pi227	YN5.48	pi227	EF5.44 YN5.48 ECB27

Figure 4.3 Markers ordered from rare recombination events as detailed in Table 4.2. *APC* represented by shaded area and site of marker locus possible at any point in the box indicated.



There is a fundamental problem with genetic linkage when the loci being tested are physically very close to each other. Recombination events are very rare in this situation and so complete ordering of markers by recombinant mapping is not always possible. However, this study has identified 12 important rare recombinant events informative at multiple loci due to the large numbers of meioses available. Linkage analysis also provides vital information for further detailed mapping of the region. The results of the analysis for linkage of *APC* to each of the 6 polymorphic DNA marker loci are presented in Table 4.3. Peak lod scores (Z_{max}) and recombination fraction (θ) indicating the maximum likelihood of marker location relative to *APC* with 95% probability limits are tabulated together with lod score tables. No recombinants have been identified between *APC* and YN5.48, or between *APC* and EF5.44. Peak lod score for *APC*--YN5.48 was 7.00 at θ=0.00, with 95% probability limits 0.00-0.07. EF5.44 was not very informative in these families and so the peak lod score for *APC*--EF5.44 was only 3.5 at θ=0.00, with resultant wide 95% probability limits.

Table 4.3 Linkage analysis in 9 Scottish FAP kindreds (KMD1-6,8,14, and 16): Peak lod score (Z_{max}) for each marker vs. *APC* at recombination fraction (θ) with 95% probability limits and lod score tables at θ= 0.00, 0.05, 0.10, 0.15, 0.20, 0.25.

Linkage vs. *APC*	Z_{max}	θ	95% probability limits	Recombination fraction (θ) 0.00	0.05	0.10	0.15	0.20	0.25
EF5.44	3.50	0.00	0.000-0.160	3.50	3.22	2.91	2.58	2.23	1.86
YN5.48	7.00	0.00	0.000-0.070	7.00	6.31	5.56	4.79	4.01	3.24
L5.62	13.31	0.02	0.005-0.095	-	12.99	11.94	10.65	9.21	7.65
ECB27	1.88	0.06	0.002-0.260	-	1.89	1.75	1.50	1.20	0.90
C11P11	5.45	0.09	0.025-0.210	-	5.23	5.43	5.13	4.59	3.90
pi227	4.40	0.11	0.035-0.235	-	4.00	4.40	4.26	3.88	3.35

Tests for linkage between each pair of marker loci revealed no discrepancies to suggest non-paternity or mis-typing. There were no cases of non-paternity on testing with the probes YNZ22 or 29C1.

Lod score calculations were carried out for linkage between each pair of markers and this is shown in Table 4.4. Recombination fraction is indicated above the diagonal and peak lod score below the diagonal for each marker-marker combination. No recombination events were detected between the following pairs of markers: L5.62--ECB27, ECB27--C11P11, C11P11--pi227 and EF5.44--L5.62. However the lod scores for EF5.44--L5.62 and for L5.62--ECB27 were low due to poor informativity of these markers in the local population.

Table 4.4 Two-point lod scores (below diagonal line) and recombination fraction (above diagonal line) for linkage between marker loci in 9 FAP kindreds.

	EF5.44	YN5.48	L5.62	ECB27	C11P11	pi227
EF5.44		0.07	0.00	0.15	0.16	0.25
YN5.48	2.11		0.11	0.19	0.11	0.25
L5.62	1.23	0.98		0.00	0.04	0.04
ECB27	0.69	0.52	1.03		0.00	0.12
C11P11	0.64	1.60	4.11	1.86		0.00
pi227	0.00	1.05	1.48	1.72	3.05	

The data presented here from lod score tables and from rare recombination events suggest that YN5.48 and L5.62 delineate a small region which spans *APC*. The interval L5.62--YN5.48 could be as much as 11cM (see Table 4.4), or 7cM (since L5.62--EF5.44 is 0cM and EF5.44--YN5.48 is 7cM), or as little as 2cM (since L5.62--*APC* is 2cM and *APC*--YN5.48 is 0cM). The latter (2cM) seems most likely since lod

scores are highest for linkage of each of the markers to *APC*. When two-point linkage analysis is added to the order derived from recombination events and given that C11P11 is centromeric to *APC* (Varesco 1989) and that YN5.48 is telomeric to *APC* (Nakamura 1988), these data support the locus order centromere--pi227--C11P11--ECB27--L5.62--*APC*--EF5.44--YN5.48--telomere. Although EF5.44 could lie in the interval L5.62--*APC* or, least likely, in the interval ECB27--L5.62.

4.4 Discussion

The genetic linkage analysis described in this chapter has provided a high resolution genetic linkage map of 6 polymorphic DNA markers which lie very close to the *APC* gene on chromosome 5q. The large number of meioses studied and the detection of a number of rare recombination events which were informative at multiple loci has allowed confident ordering of parts of the map. The possibility of double recombinations for these events is remote since the genetic area under scrutiny is very small indeed.

No *APC*-YN5.48 recombinants were detected but when the relevant two-point linkage analysis data are added into the map derived from recombination events, the localisation of YN5.48 to the other side of *APC* from pi227, C11P11, ECB27 and L5.62 is highly likely. It has been shown by *in situ* hybridisation analysis that pi227, C11P11 and ECB27 are centromeric to *APC* (Varesco 1989). Localisation of YN5.48 distal to *APC* (Nakamura 1988) now appears certain following the recent identification of an *APC*-YN5.48 recombinant which was non-recombinant for *APC*-pi227 (Tops 1989). In addition, physical mapping data (Nakamura personal communication 1990) show that EF5.44 and L5.62 are indeed around 2mB apart, supporting the linkage analysis presented here. When these data are incorporated with the recombinant data and the linkage analysis described in this chapter, a preferred locus order of centromere-pi227-C11P11-ECB27-L5.62-*APC*-EF5.44-YN5.48-telomere is strongly supported.

The present study suggests that YN5.48 and L5.62 delineate a small region of chromosome 5 which could be as little as 2cM and within this region the locus recognised by EF5.44 lies very close to the *APC* itself. This finding is a step towards the isolation of *APC* itself. It will now be possible to isolate clones from this region and screen these for the presence of expressed sequences. Any such sequences must be strong candidates for the *APC* gene itself, given the relatively small area between these markers and that *APC* is probably a fairly large gene since the rate of new mutations giving rise to sporadic cases of Familial Adenomatous Polyposis is high (Reed 1955).

Having demonstrated that EF5.44 lies very close to *APC*, it can now be used to screen pulse-field DNA fragments for rearrangements in patients with FAP and this analysis is described in Chapter 7.

The data presented here, combined with those already published for linkage of several of these markers to the *APC* gene, give lod scores sufficiently high (and hence 95% probability limits sufficiently narrow) to allow their use in presymptomatic diagnosis of FAP. The combined estimates for linkage for the markers published as lod score tables, including data from this analysis, are as follows: YN5.48-*APC*, approx. 18.5 at θ=0.025 (Nakamura 1988, Tops 1989); pi227-*APC*, 18.95 at θ=0.075 (Meera Khan 1988, Murday 1989, Tops 1989); C11P11-*APC*, 11.97 at θ=0.025 (Bodmer 1987, Leppert 1987, Murday 1989). Assessment of the practical value and the validity of the use of these markers for presymptomatic diagnosis of FAP is described in Chapter 8.

The establishment of a high density map of the region around *APC* is of great importance since, until isolation of the gene itself, multiple markers will be required for presymptomatic diagnosis of FAP. Even after the *APC* gene is cloned and sequenced, it is likely that there will be a need for linked markers in some families. This analysis has detected no evidence of genetic heterogeneity in 9 Scottish FAP kindreds, with linkage being apparent for at least one of the 6 markers studied in all families and to date there are no families in the world literature which do not exhibit linkage to 5q21-22. This is of paramount importance to clinicians who will use genetic linkage data to influence management of families and at-risk individuals under their care.

The construction of this high resolution map allows a stringent assessment of the role of deletion of the *APC* gene in the biology of familial and sporadic colorectal adenomas and carcinomas. This analysis is described in Chapter 5 and 6.

CHAPTER 5

ALLELE LOSS AND DELETION ANALYSIS IN SPORADIC COLORECTAL CARCINOMAS

5.1 Introduction

The hypothesis that a dominantly inherited gene defect might exert its effect in a recessive manner at the cellular level (Knudson 1971) and the paradigms of such tumour suppressor genes, the retinoblastoma and Wilms' tumour genes, have been discussed in Chapter 1. Solomon's demonstration of allele loss on chromosome 5 (Solomon 1987) indicated that the role of *APC* in FAP and sporadic colorectal cancer might be analogous to the situation in retinoblastoma and Wilms' tumour, implicating *APC* as a tumour suppressor gene involved in the biology, if not the genesis, of non-polyposis colorectal cancer. A search for allele loss at polymorphic marker loci close to the target locus is a powerful strategy for assessing a possible recessive determinism for *APC* in the respect that not only deletions involving *APC* can be detected, but also mitotic recombinations in the tumour tissue since the marker allele will also be reduplicated and be apparent as acquired homozygosity. However, allele loss studies using markers which are not within the target gene have an inherent disadvantage since the marker locus may be some distance from the gene of interest and may not faithfully detect all deletions or mitotic recombinations. Solomon examined the allele loss status of one of the probes (λMS8) which is a considerable distance from *APC* (Wong 1987) and so the status of *APC* itself is open to question due to the large amount of DNA between λMS8 and *APC*. Prior to the work presented here, there was no critical assessment of chromosome 5 allele loss in colorectal carcinomas and whether such loss of genetic material did indeed include *APC* itself.

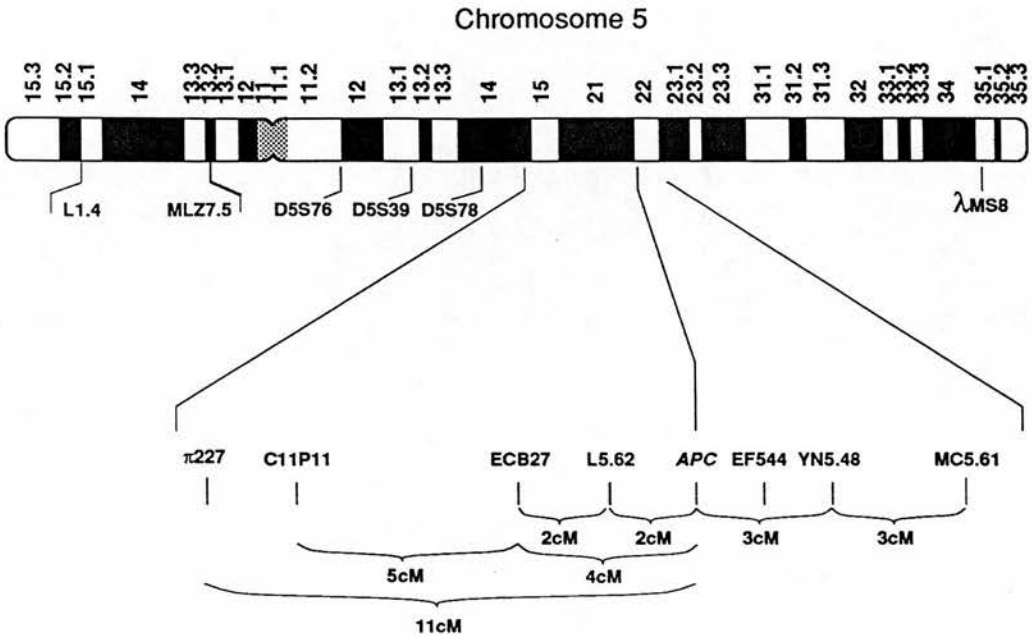
This chapter presents a detailed assessment of the allele loss status of 7 polymorphic DNA probe markers closely linked to *APC* utilising the genetic linkage map from Chapter 4 to infer the nature and extent of loss of genetic material around *APC*. A total of 13 marker loci spanning chromosome 5 have been analysed in 51 colorectal cancers in an attempt to gain greater understanding of the mechanism of *APC* deletion in colorectal cancer and of the importance of such deletions in colorectal carcinogenesis.

5.2 Methods

Fifty-one consecutive histologically confirmed sporadic colorectal carcinomas with control blood and normal colonic mucosa from 49 patients undergoing colectomy specimens were collected, processed and DNA purified as described in Chapter 2. Controls initially consisted of both leukocyte and normal mucosa DNA but after a large number of experiments it was clear there was no allele loss observed at any locus in normal mucosa and so in later experiments only leukocyte DNA served as a control.

Analysis of allele loss status at all 13 loci in all 51 cancers was assessed using paired tumour/control DNA samples as described in sections 2.6 and 2.9. Allele losses can only be are determined if the host is constitutionally heterozygous at the probed locus (ie exhibits two restriction fragment length alleles in the control blood or normal mucosa) and this results in the problem that not all tumours are informative at every locus. Tumours in which the host was constitutionally heterozygous (informative) for the markers pi227 and MC5.61 or pairs of markers internal to these loci on both sides of *APC* are referred to as informative close flanking markers. Mapping of the end of a deletion in a carcinoma by demonstration of allele losses at probed loci of known map locations is somewhat imprecise in some cases due to the inherent problem of incomplete informativity at every locus. The allele loss status for each cancer indicates the maximum size of the deletion in any given tumour but the lesion could be substantially smaller. Complete probe details are presented in Chapter 2 (Tables 2.1 and 2.2) and a schematic diagram of the localisation of the chromosome 5 marker probes which have been examined in this analysis are shown in Figure 5.1. The region around *APC* is an exploded view and the location of the 7 markers given is derived from an amalgamation of data presented in Chapter 4 and mapping data from other workers (Bodmer 1987, Leppert 1987, Meera Khan 1988, Murday 1989, Nakamura 1988, Varesco 1989). The map locations of other probes not included in the linkage map presented in Chapter 4 are referenced in Chapter 2.

Figure 5.1 Locations of 13 polymorphic loci on chromosome 5 used for deletion analysis.



Since loss of heterozygosity of markers mapping to 17p (implying inactivation of the gene for p53) has been shown to be a common event in colorectal cancer (Fearon 1987), the 17p probe, YNZ22, was also assessed for allele loss to establish whether there was any association between allele losses at 17p, 5q and clinico-pathological features. In addition, this analysis serves as a positive control to ensure that the experimental system and the population of tumours analysed were comparable with published data.

Flow cytometry was carried out on all tumours and aneuploidy was considered to be present when two distinct G_0/G_1 peaks were distinguishable, indicating two cell populations or, if there was only a single peak was visible, this was compared with the chicken erythrocyte control as described in Chapter 2.

5.3 Results

The average age of the 49 patients from whom 51 adenocarcinomas were taken was 69 years (range 29-87 years, median 58 years) and there were 2 patients under 40 years. There were one or more adenomas present in 10 of the resected

specimens (20%). Clinicopathological features of the carcinomas studied is shown in Table 5.1.

Table 5.1 Clinicopathological features of 51 colorectal cancers studied by deletion analysis. The boundary of the right and left colon has been considered to be the mid-point of the transverse colon.

Clinicopathological feature		Number	(%)
Site (n=51)			
	Caecum	9	18
	Right colon	5	10
	Left colon	4	8
	Sigmoid	16	31
	Rectum	17	33
Dukes' stage (n=50)			
	A	2	4
	B	26	52
	C	13	26
	D	9	18
Degree of differentiation (n=51)			
	Well(G1)	0	0
	Moderate(G2)	39	76
	Poor(G3)	12	24
Ploidy level (n=34)			
	Diploid	12	35
Aneuploid		22	65
	Hypodiploid	2	6
	Hyperdiploid	20	59

One carcinoma could not be fully staged as it was removed by local excision, although there were no metastases apparent on liver ultrasound scan. Unfortunately, not all carcinomas could be analysed by flow cytometry as in some cases the material had degraded probably due to a freezer thaw. The proportion of aneuploid carcinomas was made up of both hypodiploid and hyperdiploid tumours.

Allele losses were detected in many tumours and apart from 105-153Ra (D5S39), there were no marker loci at which allele loss was not detected in at least one tumour. Figure 5.2 shows an example experiment demonstrating loss of constitutional heterozygosity in carcinomas at the loci recognised by λMS8. There is a reduction in intensity or complete absence of one of the bands in those tumours which have lost constitutional heterozygosity. In some cases there is a residual faint band which represents contaminating normal stroma and lymphocytes. Figure 5.3 shows example allele losses from a number of different cancers superimposed on an idiogram of chromosome 5 which also includes a summary linkage map around *APC* as detailed in Chapter 4. The allele losses shown are all from my own series of cancers except the loss with 105-153Ra which was a tumour from the Edinburgh University series (see below).

Figure 5.2 Example experiments showing allele loss in two different carcinomas at the locus recognised by λMS8. There are two alleles in the constitutional (leukocyte) DNA and absence of one allele in each of the tumours. The residual hybridisation is due to a small amount of contaminating leukocyte and stromal DNA in the tumour. N=normal (leukocyte) DNA, T=carcinoma DNA.

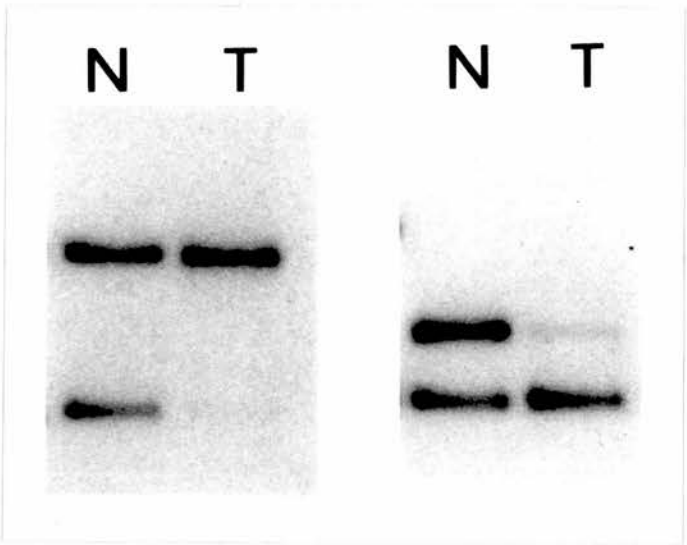


Figure 5.3 Example allele losses at each of the loci examined showing their map locations and including a simple abbreviated linkage map around *APC*.

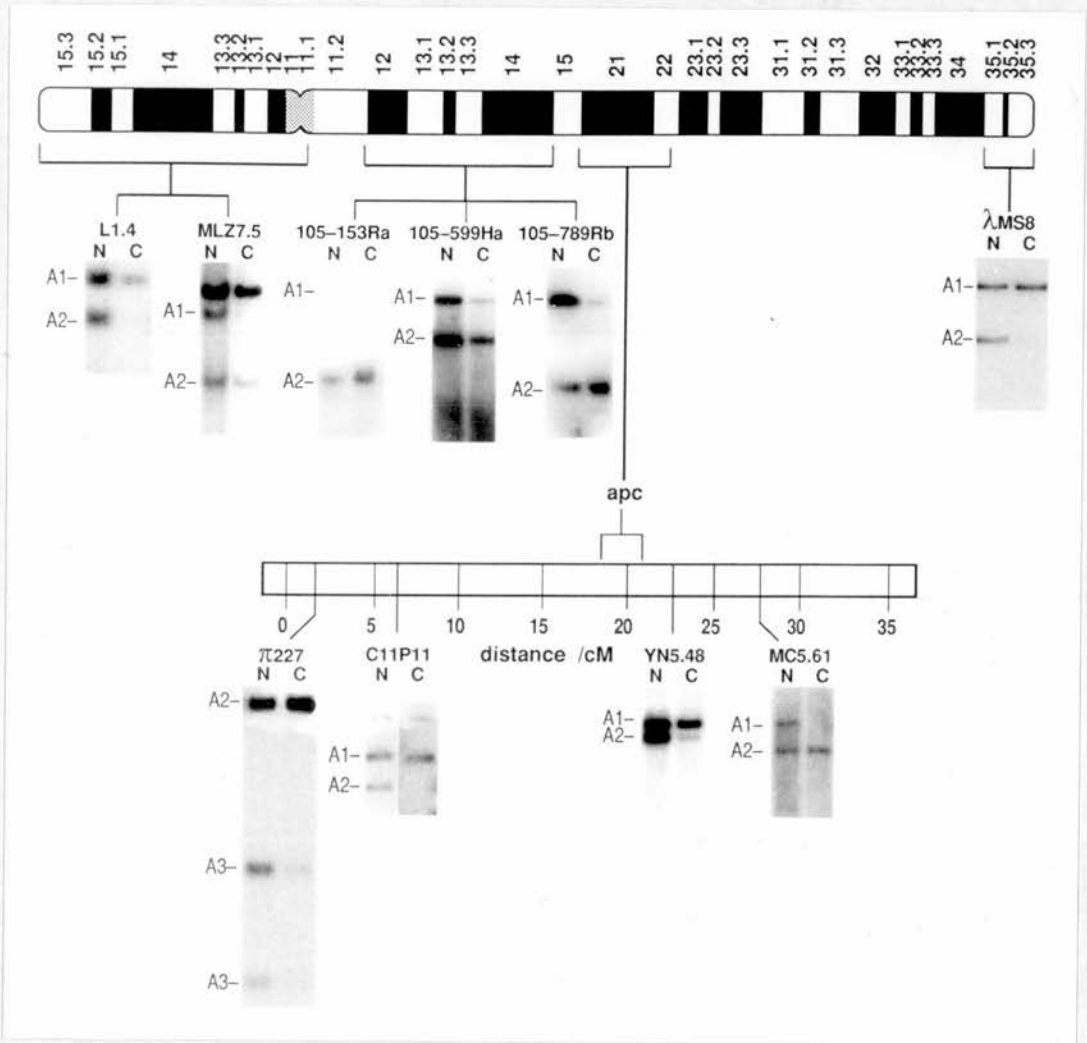


Table 5.2 shows the frequency with which allele loss was detected at each chromosome 5 locus. There appears to be a trend towards increasing frequency of detection of allele loss with increasing proximity of the marker examined to *APC* although, due to the problem of incomplete informativity, with some markers the numbers of tumours involved is small.

Table 5.2 Frequency of allele losses detected by each of the chromosome 5 probes. The number of carcinomas exhibiting loss is presented as a percentage (in parentheses) of those tumours informative at that locus. The relative order of loci are as for the linkage map in Chapter 4 and the map location of *APC* is included.

Probe informative	Carcinomas with allele loss
λMS8	13/38(34%)
MC5.61	12/21(57%)
YN5.48	9/21(43%)
EF5.44	6/11(55%)
APC	-
L5.62	3/7(43%)
ECB27	5/10(50%)
C11P11	3/11(27%)
pi227	16/35(46%)
105-798Rb (D5S78)	11/27(41%)
105-153Ra (D5S39)	0/1(0%)
105-599Ha (D5S76)	6/20(30%)
MLZ7.5	1/30(3%)
L1.4	3/19(16%)

All 51 carcinomas were informative at one or more chromosome 5 marker loci and the overall frequency of detection of allele loss at any of these loci was 34/51 (67%). The allele loss frequency when close flanking markers were examined was broadly similar with 14 of 25 cancers (56%) exhibiting loss of constitutional heterozygosity. Complete data for all 51 cancers, tabulating allele losses for all 13 probes are shown in Table 5.3. In every case apart from T132C, the allele loss patterns are consistent with loss of genetic material involving *APC* . Tumour T132C has not lost heterozygosity at L5.62 which lies between *APC* and pi227 and D5S78 which do show allele loss. This may be due to a random loss of DNA which has been shown to occur as a 'background' loss (Vogelstein 1989), or perhaps due to a local inversion-deletion within chromosome 5. Another explanation is that the map locations for the markers are incorrect. This seems highly unlikely given the confidence of the ordering shown in Chapter 4, published linkage mapping and the proportion of tumours which do have deletions consistent with *APC* as the target. Tumour T84C merits special mention since, although the deletion is consistent with *APC* involvement, the centromere also appears to have been deleted. This would not be permitted by the cell and the remaining chromosome 5q fragment should have been lost. Therefore it must be assumed that the remaining fragment has been involved in a mitotic recombination or some form of fusion to another chromosome with a centromere and this would be permitted. Tumour T188C also is of particular

interest since there appears to be a retained segment amidst an otherwise large deleted area. The pi227 locus shows no evidence of allele loss while loci either side of this (D5S78 and ECB27) do show allele loss. The reason for this must be a retained fragment of DNA (carrying the locus recognised by pi227) which has become incorporated into another chromosome or perhaps, due to its very small size, has become sequestered within the nucleus in its own right.

In many carcinomas the loss involves one side of *APC* and not the other, while in other tumours (eg. T197C2) the deleted material can be clearly be seen to involve a relatively small region of chromosome 5 which specifically includes *APC*.

Those tumours in which an area of loss of heterozygosity is clearly delineated by an area of retention of heterozygosity are referred to as interstitial deletions. Tumours where the end of the deleted region cannot be assessed with any certainty could harbour an interstitial deletion or a mitotic recombination but the methodology used here is limited because of incomplete informativity of markers. Mitotic recombination occurs at the time of malignant mitosis, early in the evolution of the neoplastic process when there is a crossing-over between chromosomal homologues with a breakpoint which must lie between the marker which shows allele loss and the one which does not. If this crossing-over, or recombination, confers a survival advantage (such as loss of a putative tumour suppressor gene) then the clone carrying the chromosome with the recombination will out-grow the other cell populations. For the purposes of this analysis, those tumours with allele loss at the tip of either arm have been considered to carry mitotic recombinations.

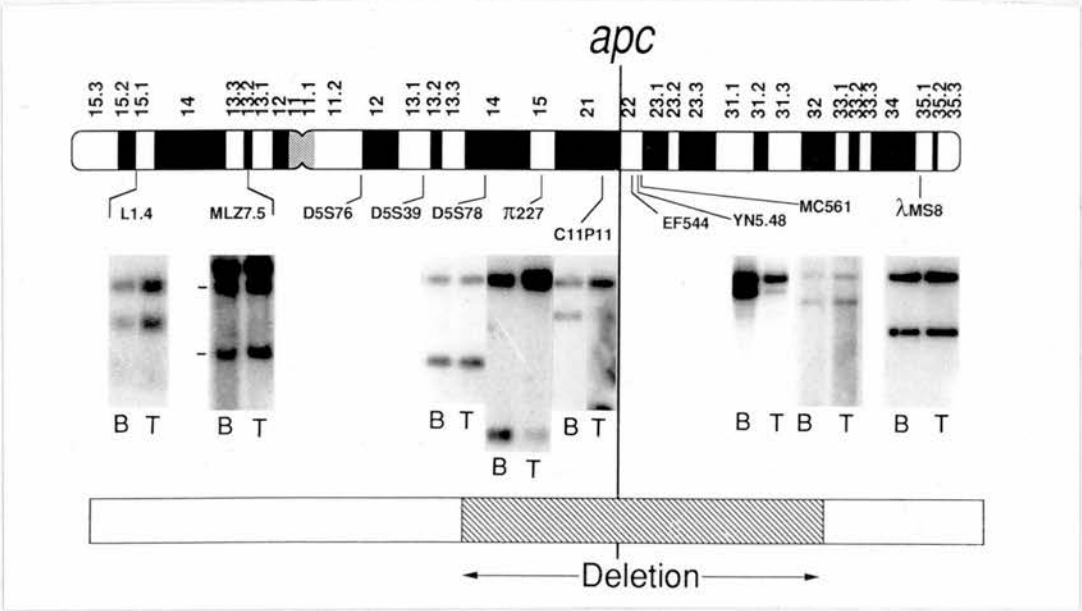
Table 5.3 Analysis of heterozygosity status for each carcinoma for loci recognised by each probe. Allele loss is represented by X, no loss and informative by O and constitutional homozygosity (non-informative) by -.

Tumour	L1.4	MLZ7.5	D5S76	D5S39	D5S78	pi227	C11P11	ECB27	L5.62	*APC*	EF5.44	YN5.48	MC5.61	λMS8
T80C	-	-	-	-	O	-	O	-	-		O	O	-	O
T83C	-	O	-	-	O	-	-	-	-		-	O	-	O
T84C	X	X	-	-	-	X	-	-	-		-	-	-	-
T85C	-	O	-	-	O	O	O	-	-		-	O	-	-
T87C	-	O	O	-	O	O	O	-	-		X	-	-	-
T94C	-	O	-	-	-	-	-	-	-		-	-	-	-
T95C1	O	O	-	-	-	X	-	X	-		-	-	-	
T95C2	O	O	-	-	-	O	-	O	-		-	-	-	
T97C	-	O	O	-	-	O	O	-	-		-	-	-	O
T98C	O	O	-	-	O	O	-	O	-		-	-	X	O
T99C	O	O	-	-	O	-	-	-	-		X	-	-	O
T100C	-	-	-	-	O	O	-	-	-		-	-	O	O
T101C	-	O	-	-	O	O	-	-	-		-	-	-	O
T102C	-	-	-	-	X	X	-	-	-		-	-	X	X
T103C	-	O	-	-	X	X	-	-	-		-	-	-	-
T104C	-	O	-	-	O	O	-	X	X		-	O	-	O
T106C	-	O	-	O	-	-	-	-	X		-	-	-	O
T109C	-	O	-	-	O	O	-	-	-		-	-	-	X
T125C	-	O	-	-	X	X	-	-	-		-	-	X	X
T130C	-	O	-	-	-	O	-	-	O		-	-	O	O
T131C	O	O	-	-	-	O	-	O	-		O	-	-	O
T132C	O	-	-	-	X	X	-	-	O		-	-	-	O
T140C	-	-	-	-	-	O	O	-	O		-	O	O	-
T141C	O	-	X	-	-	X	-	-	-		-	-	X	O
T143C	-	-	O	-	O	O	-	-	O		-	O	O	O
T144C	-	-	-	-	-	X	-	-	-		-	-	-	X
T145C	O	O	X	-	-	X	X	-	-		-	-	-	X
T151C	-	-	O	-	O	-	-	-	-		X	-	-	-
T152C	O	O	-	-	O	X	X	-	-		-	X	X	O
T153C	-	-	-	-	-	X	-	-	-		-	-	-	X
T171C	-	-	X	-	X	-	-	-	-		-	X	-	X
T177C	O	-	O	-	-	-	-	O	-		O	O	O	O
T178C	-	O	-	-	X	-	-	-	-		-	-	-	X
T181C	-	-	O	-	-	-	O	-	-		-	O	-	O
T182C	X	-	X	-	-	-	-	-	-		-	X	X	X
T187C	-	O	-	-	O	O	O	-	-		-	O	-	O
T188C	X	-	X	-	X	O	-	X	-		X	X	O	-
T189C	O	-	O	-	X	-	-	-	-		-	X	X	X
T191C	-	-	O	-	-	O	-	-	X		-	-	X	X
T192C	-	O	O	-	O	O	-	O	-		O	O	-	O
T193C	O	O	O	-	-	O	-	X	-		-	-	-	O
T194C	O	O	-	-	-	X	-	-	-		-	X	X	X
T195C	-	-	-	-	X	-	-	-	-		X	-	-	O
T196C	O	O	X	-	X	X	-	-	-		-	-	-	-
T197C1	-	-	O	-	-	X	X	-	-		-	X	X	O
T197C2	-	-	O	-	-	O	O	-	-		-	X	O	O
T198C	O	O	-	-	X	-	-	-	-		O	-	-	O
T199C	-	O	-	-	-	X	-	-	-		X	X	X	X
T200C	-	O	O	-	O	X	-	X	-		-	O	O	
T201C	O	O	O	-	-	-	-	-	-		-	-	X	O
T237C	-	-	-	-	-	-	-	-	-		-	O	O	-

The nature and extent of the loss of genetic material from chromosome 5 in any one carcinoma can be inferred in those tumours which have sufficient loci informative to delineate the ends of the deletion. This is shown diagrammatically in the example of cancer T152C in Figure 5.4. It can be seen that the deleted region must involve loss of *APC* and that the nature of that loss is interstitial deletion

involving a maximum interval of D5S78 to MC5.61 and a minimum interval stretching from pi227 to YN5.48 (around 25cM). It is should be noted that the area around *APC* is an exploded view and so the deleted region is very small in relation to the whole of chromosome 5. Therefore, somatic deletions involving chromosome 5 in sporadic colorectal cancers appear to be relatively specific for the *APC* gene.

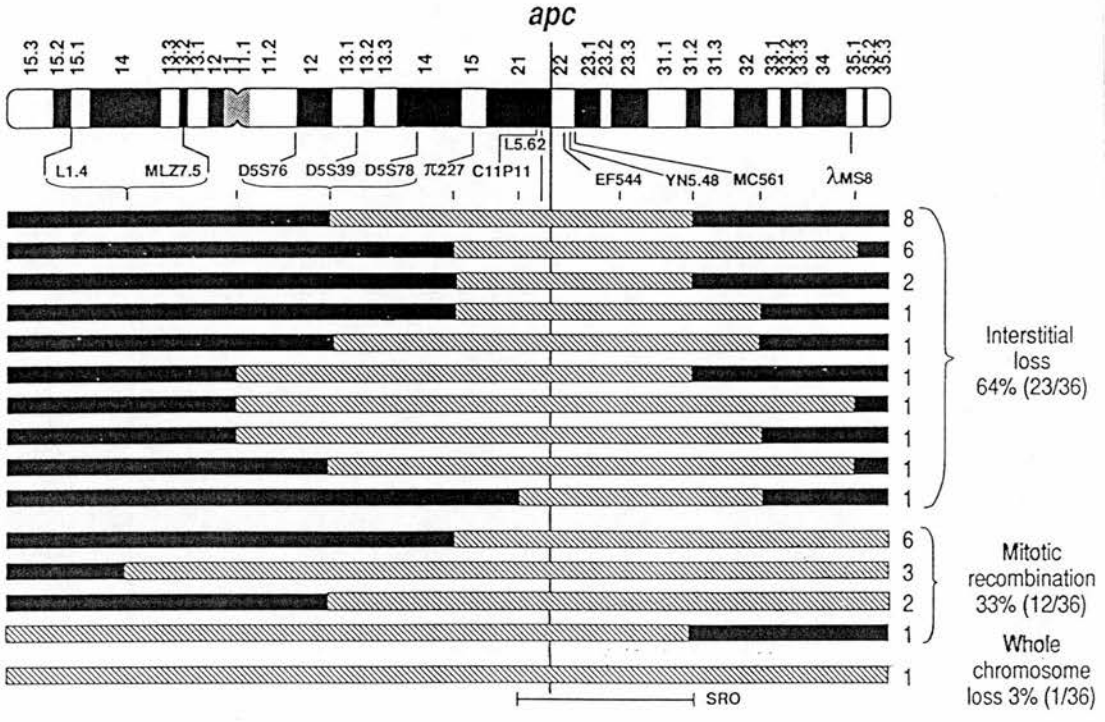
Figure 5.4 Delineation of the extent of a deletion in a sporadic colorectal carcinoma using a strategy of assessment of allele loss status at multiple chromosome 5 loci (example T152C). The maximum limits of the deleted region are represented by the cross-hatched segment.



The deletion analysis has been extended to combine all tumours with sufficient markers informative to delineate the extent of the loss of genetic material into a composite deletion map. Due to the analysis of 13 markers in each of my own series of tumours it was possible to characterise the deletion in 24 tumours (see Table 5.3). Incomplete probe informativity reduced the number of tumours giving data sufficient for deletion mapping and so data from my co-workers in Edinburgh University have been included to strengthen the data for my own series (see Table 5.2). Using the same battery of probes, 104 colorectal carcinomas from Edinburgh University Department of Pathology were analysed in parallel, although 13 markers were not assessed in every case, as in my own series. Experimental technique was uniform for the two data sets and is described above. In all there were 36 tumours

of the first 119 combined tumour data set with sufficient informative markers to allow construction of a composite deletion map. These are shown in Figure 5.5 and it can be seen that there is a relatively specific deletion of *APC* which is usually due to interstitial deletion (64%) and only rarely due to whole chromosome loss. Cancer T132C has been excluded as it clearly is an exception to the observation in all other tumours that the deletion consistently involves *APC*.

Figure 5.5 Composite deletion map derived from 36 colorectal carcinomas with sufficient markers informative to allow delineation of the nature and the extent of the loss of genetic material from chromosome 5. SRO= smallest region of deletional overlap.



Allele losses were distributed evenly throughout the different clinico-pathological classes of carcinomas (Table 5.4). There was no predilection for *APC* inactivation (as demonstrated by the occurrence of allele losses at any informative 5q locus or at close flanking loci) with respect to any particular site, Dukes' stage, ploidy level or degree of differentiation. Notably, both Dukes' stage A tumours exhibited loss at 5q and this supports the notion that *APC* inactivation is an early event and precedes tumour progression.

Table 5.4 Allele loss frequencies at any informative 5q marker, *APC* close flanking markers and YNZ22 classed by site, Dukes' stage, ploidy level, degree of differentiation and the presence of 17p allele loss. The numerator in each case is the number with loss and the denominator the number informative for those markers .

Cancer status	Allele loss frequency at marker(s) informative		
	Any 5q	Close flanking	YNZ22
Site			
Caecum	4/9(44%)	3/7(43%)	5/8(63%)
Right	5/5(100%)	1/1(100%)	1/5(20%)
Left	2/4(50%)	0/2	1/3(33%)
Sigmoid	12/16(75%)	7/9(78%)	9/12(75%)
Rectum	11/17(65%)	3/6(50%)	9/13(69%)
Dukes' Stage			
A	2/2(100%)	-	-
B	14/26(54%)	7/15(47%)	14/22(64%)
C	10/13(77%)	3/5(60%)	7/11(64%)
D	8/9(89%)	4/5(80%)	3/6(50%)
Ploidy			
Diploid	7/12(58%)	1/4(25%)	8/12(67%)
Aneuploid	15/22(68%)	9/14(64%)	9/16(56%)
Degree of differentiation			
Well	-	-	-
Moderate	25/39(64%)	8/17(47%)	20/31(65%)
Poor	9/12(75%)	6/8(75%)	5/10(50%)
17p allele loss			
Loss	16/25(64%)	8/12(67%)	-
No loss	10/16(63%)	2/9(22%)	-

Data derived from experiments using the probe YNZ22 to detect loss of genetic material from chromosome 17p are also shown in Table 5.3. Allele losses detected by YNZ22 occurred in 25 of the 41 (61%) informative carcinomas and there was no tendency for 5q allele loss and 17p allele loss to occur in the same tumours. The majority of tumours had lost alleles at one or more loci with 16 (31%) having lost 17p and 5q alleles, 29 (57%) having lost either and only 6 (12%) having lost neither. Those tumours with both 5q and 17p deletions were not of any particular

Dukes' stage (8 stage B, 5 stage C and 3 stage D) and were of similar ploidy levels (6 aneuploid and 4 diploid) and so there does not appear to be a progressive accumulation of genetic defects associated with poor prognosis in this data set. The data do suggest that loss of *APC* and p53 take place independently of each other and of all features currently classed as reflecting tumour progression. One previous report has suggested a strong polarisation of allele losses towards the tumours in the distal colon (Delattre 1990) but the data presented here do not support this (see Table 5.3), although the numbers of tumours analysed for this thesis and presented here are relatively small. Even when caecal and right colonic tumours are classed as proximal lesions and all others as distal, the allele loss frequencies remain similar (9/14 (64%) proximal, 25/37 (68%) distal lost any 5q marker; 6/13 (46%) proximal, 19/28 (68%) distal lost YNZ22). Ploidy levels were also similar in proximal and distal tumours (5/11 (45%) analysable proximal tumours and 8/15 (53%) distal tumours were aneuploid .

5.4 Discussion

The data set of colorectal cancers presented here appears to be broadly similar to reported series with respect to the age of the affected patients (Falterman 1974), tumour stage (Dukes' 1929-30 and 1940, Falterman 1974, Gill 1978, Jarvinen 1988, Phillips 1984), degree of differentiation (Jass 1987), topographical distribution (Falterman 1974, Jarvinen 1988), DNA ploidy level measured by flow cytometry (Armitage 1985, Jones 1988) and the frequency of detection of loss of genetic material from chromosome 17p (Baker 1989, Fearon 1987, Law 1988, Lothe 1988, Monpezat 1988, Okamoto 1988, Sasaki 1989, Vogelstein 1988). Therefore in all these respects, this collection of tumours is similar to other non-selected groups. However, the frequency of detection of *APC*-related allele loss is substantially higher than in published reports (Law 1988, Okomato 1988, Sasaki 1989, Solomon 1987, Vogelstein 1988, Wildrick 1988). This discrepancy is due entirely to the use of markers mapping very close to, and flanking, the *APC* gene. The frequency of detection of allele loss *at each* locus in this study is similar to, or in some cases actually lower than, published allele loss frequencies using the respective probe, thus Solomon detected 40% λMS8 loss compared to 34% in this series and Okamoto detected 33% loss at C11P11 compared to the 27% shown here. In the case of sporadic retinoblastoma, a similar increased frequency of detection of allele losses was found when markers closely flanking the Rb-1 gene on chromosome 13q14 were assessed (Dryja 1986). The frequency of detection of allele losses with further markers which map still closer to *APC* might therefore be higher still and it is likely

that some lesions will confined to *APC* itself. A further reason for the increased frequency of *APC* related allele loss found here is the density of markers used. In almost every case, each tumour has been assessed for allele loss at 13 loci spanning the whole of chromosome 5. As shown in Figure 5.5, interstitial deletions and mitotic recombinations can extend in either direction from *APC* and so previous studies which rely on the assessment of allele loss at one locus, on one side of *APC* will undoubtedly miss a number of deletions which involve a considerable portion of chromosome 5 on the opposite side of *APC*.

The technique employed in this study for detection of allele loss involves an element of subjective assessment of the relative intensities of each of the alleles revealed on the X-ray film by P^{32} labelled probe. There is potential for mis-classifying tumour allele loss status due to the presence of small amounts of residual normal tissue and lymphocytes which reflect the host's constitutional heterozygosity. Initially laser densitometry was employed to introduce more objectivity to the assessment of the relative intensity of each allele. Unfortunately this objectivity is not practically achievable since the level of diminished intensity set on the laser densitometer is set originally on an arbitrary, subjective assessment of the level of allele intensity difference which represents allele loss. Therefore laser densitometry was abandoned and visual comparison of relative allele intensities employed throughout and this is the standard method now used by most other authors. In the majority of cases, there was no dubiety when allele loss was present or absent. In those cases where there was some doubt, an independent assessment was obtained and if no agreement could be reached, then the tumour was classed as not informative at that locus. To overcome the problem of contamination, some authors have recommended section and microdissection of the cancer tissue to separate it from adjacent normal or benign tissue (Vogelstein 1988) while others have suggested separation of cell populations within any given tumour by flow cytometry· (Monpezat 1988). However, these methods are extremely time-consuming, require resources outside the scope of this project and provide little advantage over the method used here. Sampling a macroscopically representative malignant portion of tumour tissue immediately adjacent to the sections taken for routine histological assessment has ensured that the tissue being analysed is indeed mostly carcinoma. The validity of this strategy is demonstrated by the fact that the frequency of allele loss with probe YNZ22 in this series is very similar to those reports using selectively isolated tumour tissue (Monpezat 1988, Vogelstein 1988) and indeed 5q allele losses are much higher than in other reports. Contamination of cancer tissue with benign or normal tissue could only have the effect of reducing the apparent frequency of allele loss at any locus. The similarity of YNZ22 allele loss

frequency in this study to reported series also serves as an independent control and suggests that the experimental technique involving the extraction and hybridisation of tumour DNA with probe DNA is sound.

This study has utilised the linkage map of the region around *APC* presented in Chapter 4 to allow the construction of the first detailed deletion map of the chromosomal region around *APC* in colorectal carcinoma. The identification of a large number of markers very close to *APC* and ordering of these relative to each other and to *APC* has been fundamental to this deletion analysis. The work presented here has shown that the mechanism of *APC* loss is predominantly due to interstitial deletion and only rarely due to whole chromosome loss, in contrast to some other tumours (Hansen 1988, Ponder 1988). A very limited 5q deletion map in colorectal cancer has been published (Vogelstein 1988) and this supports the findings presented here that interstitial deletion is a frequent finding. The chromosome 5q deletions shown in Table 5.2 are also highly specific for *APC*, with the deletion involving *APC* in all but one case. This discrepancy may be due to rearrangement of a portion of chromosome 5 which includes *APC* in this tumour or may be due to non-specific 'background' loss of genetic material which may well occur at a level of around 10% (Vogelstein 1989).

These data demonstrate that deletions can extend in either direction from *APC* (telomeric or centromeric). This has important implications for mapping of *APC* itself. Linkage mapping becomes ever more laborious as markers closer to the gene of interest are identified since recombinations occurring in the very small interval between become ever less frequent. Deletion mapping in cancers could hold the key to final isolation of *APC*. If, in one tumour, a retained marker can be shown to delineate the centromeric end of a deletion extending towards the telomere, while in another tumour that same marker is retained and delineates the telomeric end of a deletion which extends towards the centromere, then that marker must be very close to, if not within, *APC* itself. This is currently one of the strategies being employed by Dr. Yusuke Nakamura in the search for *APC*.

The data presented in this chapter leaves little doubt that the target gene on chromosome 5 is indeed *APC*. Unless there is some unrelated, coincidental predisposition to genetic alterations in this region, loss of *APC* must confer growth advantage on affected clones and result in positive selection for a cell population which is *APC*-deleted.

The reason for interstitial deletion rather than whole chromosome loss as the predominant mechanism of *APC* loss cannot be explained by these, or any published data but merits some discussion. One hypothesis is that hemizygous loss of the whole of chromosome 5 (which is a relatively large chromosome) would result in

haploid insufficiency of sufficient genes which are important to cell survival that the result would be cell lethal. However, clearly some tumours do lose the whole chromosome and so this cannot be the whole explanation. Another possibility is that one or a number of crucial genes are already in the haploid state in the region around *APC*. The net result of deletion of the remaining homologue would be homozygous deletion of these important genes, which could not be sustained. This scenario allows for the occurrence of both whole chromosome loss and of interstitial deletions.

Genes located close to *APC* (and indeed *APC* itself, see discussion in Chapter 6) might be rendered haploid due to random somatic or constitutional mutations which are known to occur fairly frequently. However, unless there is a specific mutational hotspot around this region, this explanation seems unlikely. Another, more attractive mechanism by which haploid inactivation could be achieved is by the phenomenon of genomic imprinting. Genomic imprinting is due to differential modification of the phenotype of any given gene depending on its parental origin (Editorial, Genes and Dev. 1989, Reik 1989, Sapienza 1989). It has been suggested that genomic imprinting is common and may affect large numbers of genes (Sapienza 1989). Imprinting may be due to differential parent-of-origin mediated methylation or to DNA packaging and is usually only implicated as showing an effect during embryogenesis (Editorial, Genes and Dev. 1989). However, data from sporadic osteosarcoma (Toguchida 1989) suggest that, while imprinting certainly exerts effects on recessive embryonal cancer genes (Reik 1989, Scrable 1989), these effects are not solely manifest in childhood. Hence genomic imprinting may result in functional haploid inactivation of a number of genes in the region of *APC*. Genotypic inactivation of the residual functioning copy of one of these genes by somatic hemizygous loss would be cell lethal and so there would be selection against such an occurrence. However, a large deletion affecting a number of genes would be allowable if those genes were already functionally in the haploid state due to imprinting. This hypothesis would explain the findings presented in this chapter with regard to the variable extent of somatic deletions seen in colorectal cancer tissue and the fact that both interstitial deletions and whole chromosome loss both occur. The number and location of imprinted genes close to *APC* will determine the allowable mechanism of *APC* loss. The extent of the involvement of genomic imprinting in the *APC* deletions will become testable once *APC* is cloned and intragenic defects which occur in colorectal cancer has been established.

Another explanation for the occurrence of substantial hemizygous losses of chromosome 5q could be due to mitotic recombination or whole chromosome loss by non-disjunction and chromosomal reduplication. The net result of both of these

mechanisms is reduplication of the mutant apc with the maintenance of the diploid state of the genes necessary for cell survival. Gene dosage studies would allow an experimental test of this notion by comparing tumours with interstitial deletions to those with mitotic recombinations. Tumours with acquired homozygosity at apc-linked marker loci should exhibit a doubling of the intensity of the retained allele if allele loss was due to recombination or non-disjunction with reduplication, whereas such a doubling of allele intensity would not be apparent if the observed allele loss were due to interstitial deletion. However, this series of experiments was not within the scope of this project.

The data presented here suggest that inactivation of *APC* occurs independently of all of the parameters currently thought to reflect tumour progression, implying that *APC* loss occurs early in colorectal carcinogenesis. If the FAP syndrome is considered to reflect the stages of colorectal tumour initiation and progression then it is tempting to propose that apc lesions may actually be implicated as one of perhaps a small number of possible causative events. The timing of apc deletions in the adenoma-carcinoma sequence is discussed further in Chapter 6. However, it is certainly reasonable to propose that apc inactivation is not involved in the later stages of metastasis and invasion since there is no increased frequency of 5q allele losses in more advanced tumours. The data presented here show no increase in the frequency of 17p allele loss in the more advanced tumours but such allele loss is very common and the number of informative tumours was relatively small. However, in one study 17p allele loss was shown to parallel tumour progression but 5q allele loss did not (Delattre 1990). Therefore, the data presented here and all published data concur in suggesting that *APC* inactivation is an early event in the progression of normal colorectal mucosal cells along the pathway to malignancy. It would be of great interest to know of the survival of patients who had tumours with apc deletions and those with p53 deletions to assess whether such lesions do predetermine outcome. One would expect that *APC* loss should not if it is indeed implicated in the genesis of colorectal cancer but the loss of p53 implied by YNZ22 loss may well provide a prognostic indicator, independent of those currently in use.

These data are the first to show the true high frequency of *APC* loss in colorectal cancer tissue and to delineate the nature and extent of that loss. Clearly, there is still a lot to learn about the involvement of *APC* in colorectal carcinogenesis.

CHAPTER 6

ALLELE LOSS IN FAMILIAL AND SPORADIC ADENOMAS.

6.1 Introduction

Allele loss analysis presented in Chapter 5 has demonstrated consistent and specific deletions of the *APC* gene in colorectal carcinomas. This chapter addresses the question of whether such allele losses can be detected in adenomas arising sporadically or in patients with FAP. It would then be possible to assess whether the inherited *APC* gene mutation, which affects only one gene copy, can allow adenomatous change in the colonic epithelium of FAP patients or whether a second, somatic, event is required. It might also be possible to gain some measure of the timing of *APC* gene inactivation in the adenoma-carcinoma sequence in the sporadic case.

A series of observations is presented which constitute a stringent assessment of the deletion status of the *APC* gene in FAP and non-FAP adenomas. Employing mapping data from Chapter 4, multiple polymorphic DNA markers have been utilised to examine allele loss patterns at 4 polymorphic loci close to, and flanking, *APC* in FAP adenomas and at a total of 13 polymorphic loci spanning chromosome 5 in sporadic adenomas. Comparison of the frequency of 5q allele loss in FAP and sporadic adenomas and between sporadic adenomas and carcinomas has been made both locus-by-locus and by assessing allele loss frequency detected by use of close flanking markers.

Previous reports have shown 17p allelic deletions in colorectal cancers and sporadic adenomas (Lothe 1989, Sasaki 1989, Vogelstein 1988). This implies loss of the gene for p53 which was originally thought to be an oncogene (Oncogene editorial 1988) but has subsequently been shown to have tumour suppressor activity (Finlay 1989). Hence an analysis of allele loss using a highly polymorphic 17p marker (YNZ22) is also presented to compare the frequency of such loss in this series of tumours.

6.2 Methods

6.2.1 Clinical material.

Forty adenomas were harvested from the resected specimens of 4 patients with FAP undergoing prophylactic colectomy, having been identified as gene carriers by the screening program presented in Chapters 3 and 8. A total of 20 sporadic adenomas were harvested from 14 colectomy specimens resected because of co-existing carcinoma or because the adenoma was large and/or symptomatic. The 51 sporadic colorectal carcinomas detailed in Chapter 5 serve for comparison. Peripheral venous blood samples were obtained from all patients.

All tumours were placed immediately on ice and tumour size assessed in the fresh state by measurement of maximum transverse diameter. Adjacent portions of tumour were taken for DNA analysis, histology and flow cytometry as described in Chapter 2. All sporadic adenomas and all FAP adenomas >5mm were examined histologically after fixing, paraffin-embedding and H and E staining. The remaining FAP polyps were macroscopically adenomatous with no macroscopic evidence of malignancy but, due to the small size of these lesions, they were not all examined histologically. Great care was taken to avoid contamination of polyp tissue with any surrounding normal mucosa in all classes of tumour. Normal colonic mucosa and peripheral blood leukocytes once again served as controls. For FAP adenomas, peripheral blood leukocyte DNA from each patient served as a control since all of the colonic epithelium can be considered abnormal.

DNA purification and analysis was as described in sections 2.5, 2.6 and 2.9.

6.2.2 DNA probes

Most FAP adenomas were small and so the number of probes used to assess allele loss was limited to the following 4 probe/enzyme combinations in order to conserve limited amounts of invaluable DNA: C11P11(Taq1), L5.62(BglII), EF5.44(Msp1) and YN5.48(Msp1). Additional chromosome 5 probes used for analysis of sporadic tumours were : 7MS8(Hinf1), MC5.61(Msp1), 105-798Rb(Msp1), 105-153Ra(Msp1), 105-599Ha(Taq1), L1.4(EcoR1) and MLZ7.5(EcoR1). Chromosome 17p allele loss was assessed using YNZ22(Taq1) in familial and sporadic neoplasms. Further probe details, allele sizes and polymorphism frequencies are shown in Chapter 2.

6.2.3 Deletion analysis

Assessment of heterozygosity status of the polymorphic markers described in Chapter 5 in the analysis of sporadic carcinomas was also carried out for adenomas. Criteria for classification of allele loss and the definition of informative close flanking markers were as before.

Chapter 5 demonstrates that in carcinomas, loss of heterozygosity does not occur in all tumours and only a proportion of tumours are informative at any given probe locus. Therefore, statistical analysis has been carried out to assess whether variations in allele loss frequency constitute real differences which are a function of the different classes of tumour examined. Statistical tests used were X^2 analysis with Yates's correction and Fisher's exact test where appropriate.

6.2.4 Flow cytometry.

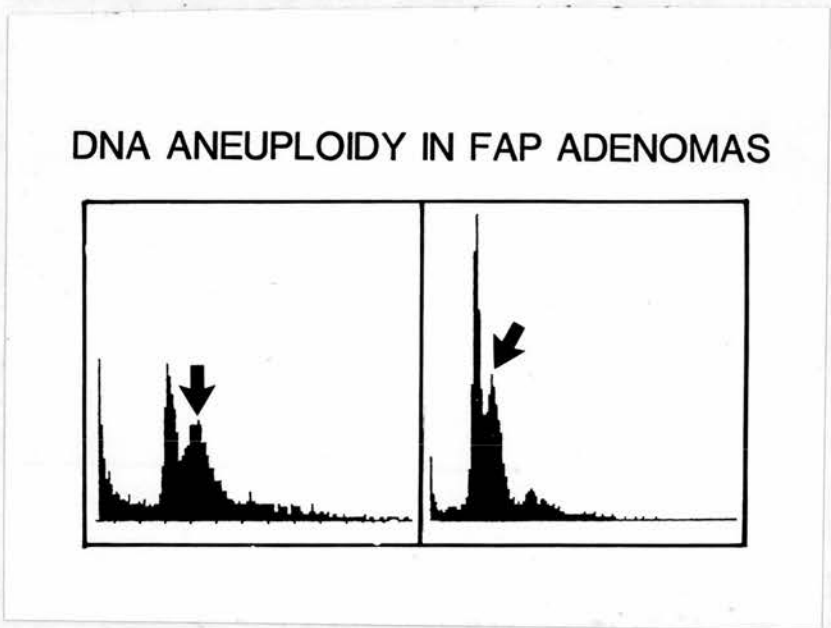
Flow cytometry, as described in Chapter 2, was carried out on FAP and sporadic adenomas to assess the overall ploidy level in order to relate this to 5q allele loss frequency.

6.3 Results

6.3.1 Assessment of allele loss in FAP adenomas.

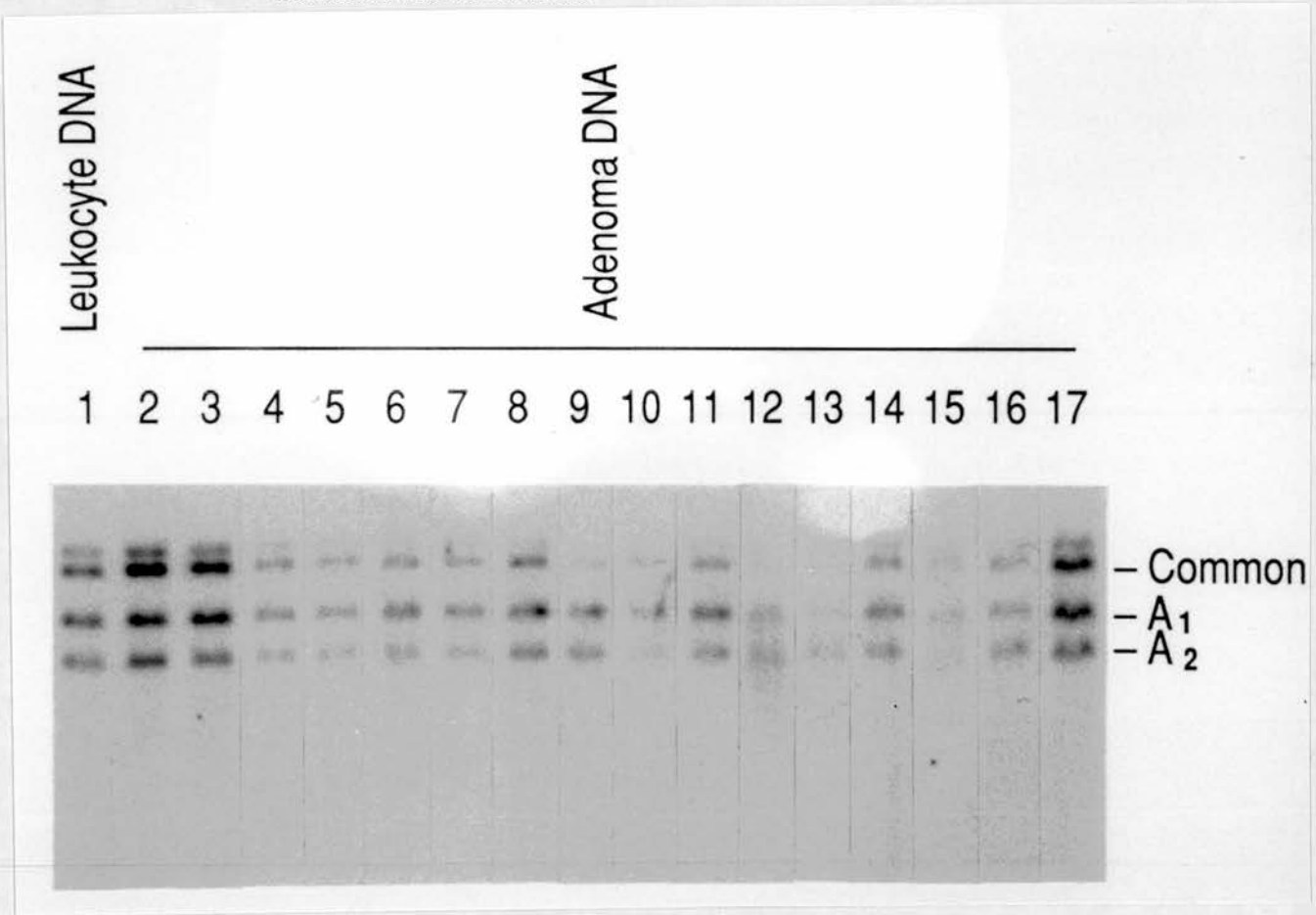
FAP adenomas ranged in size from 3mm to 1.4cm with only two adenomas being over 1cm. Due to the precious nature of DNA from these tumours, flow cytometry was only carried out on the larger adenomas. Despite their small size, all adenomas tested were aneuploid (example tracings shown in Figure 6.1). In addition, histological examination of even small adenomas revealed quite aggressive cytological characteristics (see extreme example in Figure 3.1d).

Figure 6.1 Example flow cytometric tracings from 2 FAP adenomas. There is an aneuploid population (arrowed) with the diploid peak due to contaminating stroma or lymphocytes combined with a sub-population of diploid neoplastic cells.



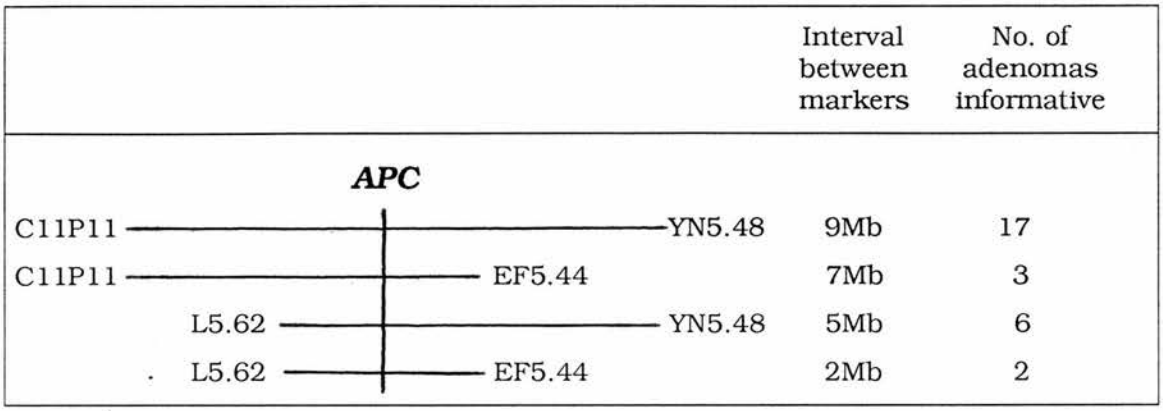
Twenty-eight FAP adenomas were informative and gave satisfactory technical results with one or more of the 4 probes used in this analysis. There was no loss of heterozygosity in 28 adenomas informative at C11P11, 17 informative at YN5.48, 8 informative at L5.62 and 3 informative at EF5.44. Figure 6.2 shows a representative analysis of 16 adenomas from a patient (KMD 1/5-17) using probe C11P11 in a Southern blot and DNA hybridisation experiment and demonstrates that there was no deletion involving the locus recognised by C11P11.

Figure 6.2 Representative experiment showing no loss of constitutional heterozygosity at the locus recognised by probe C11P11 in 16 adenomas from a patient with FAP who was informative with C11P11. Peripheral blood leukocyte DNA served as control DNA. A1 indicates the 4.4kb allele, A2 the 3.9kb allele and a common, non-polymorphic doublet is also indicated.



In total there were 20 FAP adenomas informative on *both* sides of *APC* and no allele loss was detected in any of these tumours. The number of FAP adenomas informative and the interval spanned for each pair of flanking probe loci are shown in Figure 6.3. All of these probes map very close to the *APC* gene and so the analysis of such markers informative on *both* sides of *APC* affords a highly stringent analysis of the deletion status of that gene.

Figure 6.3 Combinations of marker loci and the interval delineated by these markers in FAP adenomas with informative close flanking markers (n=20). No loss of heterozygosity was demonstrated at any of these loci.



	Interval between markers	No. of adenomas informative
C11P11 — YN5.48	9Mb	17
C11P11 — EF5.44	7Mb	3
L5.62 — YN5.48	5Mb	6
L5.62 — EF5.44	2Mb	2

6.3.2 Assessment of 5q allele loss in sporadic adenomas.

Allele loss was detected in 3 of the 18 sporadic adenomas (17%) informative at any chromosome 5 marker and 1 of the 7 adenomas (14%) with informative close flanking markers. Sporadic adenomas ranged in size from 0.5cm-5.5cm and the allele losses were distributed throughout the size range, occurring in adenomas of 0.5cm (tubular adenoma), 2.8cm (tubulovillous adenoma) and 1.0cm (tubulovillous adenoma). Data on allele loss status for all sporadic adenomas are shown in Figure 6.4. This analysis gives a qualitative indication of loss of genetic material in sporadic adenomas and the data are consistent with involvement of the *APC* gene in every case. Since markers mapping considerable distances from *APC* also exhibited allele loss (eg T150A), the extent of the deletions in adenomas seem to be similar to that seen in the carcinomas presented in Chapter 5.

Figure 6.4 Allele loss analysis in 18 sporadic adenomas informative at 1 or more of the 13 polymorphic loci. X=allele loss, O=no loss, -=not informative. Maximum diameter is given for each adenoma in the fresh state.

Tumour	Size (cm)	L1.4	MLZ7.5	D5S76	D5S39	D5S78	pi227	C11P11	ECB27	L5.62	*APC*	EF5.44	YN5.48	MC5.61	λMS8
T84A	0.75	O	O	-	-	-	O	O	-	O		-	-	-	-
T87A	1.0	-	O	O	-	O	O	O	-	-		O	-	-	-
T105A	1.0	-	-	-	-	-	-	-	-	-		-	-	O	O
T131A	0.5	O	O	-	-	-	O	-	O	-		X	-	-	X
T140A	1.8	-	-	-	-	-	O	O	-	O		-	O	O	-
T141A	0.5	O	-	O	-	-	O	-	-	-		-	-	O	O
T150A	2.8	-	-	X	-	-	-	-	-	-		-	-	-	-
170A	2.0	-	-	-	-	-	O	-	-	-		-	-	-	O
T180A	2.0	O	-	-	-	O	O	-	-	-		-	O	O	O
T191A1	4.0	-	-	O	-	-	O	-	-	-		-	-	O	O
T190A2	0.6	-	-	O	-	-	-	-	-	-		-	-	O	O
T192A1	1.8	-	O	O	-	O	O	-	O	-		O	O	-	O
T192A2	0.7	-	-	O	-	-	-	-	-	-		-	-	-	O
T237A1	5.0	-	-	-	-	-	-	-	-	-		-	O	O	-
T237A2	5.5	-	-	-	-	-	-	-	-	-		-	O	O	-
T237A3	1.0	-	-	-	-	-	-	-	-	-		-	X	O	-
T237A4	2.0	-	-	-	-	-	-	-	-	-		-	O	O	-
T237A6	3.5	-	-	-	-	-	-	-	-	-		-	O	O	-

Due to the confounding biological problem that not all individuals are informative for every probe, an assessment of whether FAP and sporadic adenomas represent 2 separate populations as regards the *APC* deletion status has to be based on statistical comparison of allele loss frequencies in the two classes of tumour. The small numbers of adenomas in this series preclude valid comparison of allele loss frequencies in sporadic adenomas and carcinomas and so the analysis presented here has been expanded to include a further set of sporadic adenomas analysed by my co-workers in Edinburgh University Pathology Department. The same battery of probes were employed in both data sets and the following comparison combines my own data on 18 informative sporadic adenomas and 28 informative FAP adenomas with data on a further 40 informative sporadic adenomas analysed in Edinburgh University.

There was a significant difference in allele loss frequency at any chromosome 5 locus between sporadic adenomas (18/58, 31%) and FAP adenomas (0/28) (X^2=9.195, $p<0.01$). However, a more critical analysis of this difference is by comparison of allele loss frequency at close flanking markers in tumours of similar size (ie. <1.5cm), since size may be a predictor of progression to malignancy (Muto 1975). Six of 12 sporadic adenomas (50%) in this category and none of 20 FAP adenomas exhibited such *APC* related allele loss (X^2=9.24, $p<0.01$).

The numbers of tumours analysed have been expanded by the Edinburgh University series to allow assessment of the relative contribution of the allele loss

frequency at each of the 4 loci examined in FAP adenomas compared to sporadic adenomas (Table 6.1). Despite this analysis being larger than any other published to date, the problem of incomplete informativity mean that the trend observed in each case just fails to reach statistical significance.

Table 6.1 Frequency of allele loss detected at each of the 4 loci examined in FAP adenomas compared with sporadic adenomas when analysed at respective loci. The overall number of each category of tumour analysed (not the number informative) is given in parentheses.

Probe	Familial adenomas (n=40)	Sporadic adenomas (n=65)
YN5.48	0/17(0%)	4/22(18%)
EF5.44	0/3(0%)	4/14(29%)
L5.62	0/8(0%)	2/12(17%)
C11P11	0/28(0%)	0/3(0%)

6.3.3 Assessment of 5q allele losses in sporadic adenomas and carcinomas.

The frequency of detection of chromosome 5 allele losses in the 18 informative sporadic adenomas presented here was compared to the frequency of allele losses detected in sporadic carcinomas as presented in Chapter 5. There was a statistically significant difference in allele loss frequency detected at any informative chromosome 5 locus between sporadic adenomas and carcinomas; 3 of 18 informative adenomas (17%) and 34/51(67%) of informative carcinomas (X^2=11.44, p<0.001; exact probability, p<0.0006). There is no bias in these data due to carcinomas being informative more often and at loci closer to *APC* than the adenomas since the mean number of informative markers mapping <15mB from *APC* (ie those at or internal to pi227 and MC5.61) in adenomas was 2.9 (range 1-5) and in carcinomas was 2.27 (range 1-5). Tumours with informative close flanking markers were therefore compared and the difference in frequency of allele losses detected in adenomas (1/7, 14%) and carcinomas (14/25, 56%) is maintained although this fails to reach the level of conventional statistical significance (X^2=2.33, exact probability p=0.12).

Comparison of allele loss at each locus in sporadic adenomas and in carcinomas is shown in Table 6.2. Data from probes 105-798Rb, 105-153Ra and 105-599Ha have been pooled, as have data from L1.4 and MLZ7.5, as these markers

map very close to each other and are some considerable distance from *APC* (Leppert 1987).

Table 6.2 Allele loss frequency 5 polymorphic DNA markers in sporadic adenomas and carcinomas. Number of tumours informative with those exhibiting allele loss at each locus (percentage in parentheses).

Probe location	Informative probes	Allele loss frequency Adenoma (n=20)	Allele loss frequency Carcinoma (n=51)
-	Any chromosome 5	3/18(17%)	34/51(67%)*
-	Close flanking	1/7(14%)	14/25(56%)
5q34	λMS8	1/9(11%)	13/38(34%)
	MC5.61	0/11(0%)	12/21(57%)**
	YN5.48	1/8(13%)	9/21(43%)
	EF5.44	1/3(33%)	6/11(55%)
5q21-23	L5.62	0/2(0%)	3/7(43%)
	ECB27	0/2(0%)	5/10(50%)
	C11P11	0/3(0%)	3/11(27%)
	pi227	0/9(0%)	16/35(46%)
	105-798Rb		
5q13	105-153Ra	1/8(13%)	14/38(37%)
	105-599Ha		
5p	L1.4	0/6(0%)	3/36(8%)
	MLZ7.5		

*X^2=11.44, p<0.001; **X^2=7.76, p<0.01.

The trend towards a lower frequency of loss of heterozygosity at each locus in sporadic adenomas when compared with carcinomas supports the overall data on any informative marker although this difference only achieves statistical significance with the probe MC5.61. There were no cases of homozygous deletion detected with any marker in colorectal adenomas.

6.3.4 Assessment of 17p allele losses in FAP and sporadic neoplasms.

There was no YNZ22 allele loss detected in the 28 FAP adenomas which were informative with this probe. Good technical results were only obtained for 15 sporadic adenomas and 1 of the 8 (13%) informative with YNZ22 exhibited 17p allele loss (Table 6.3). When the small numbers of tumours analysed are considered, the frequency of allele loss is broadly similar to that reported in other studies (Lothe 1989, Sasaki 1989, Vogelstein 1988). As detailed in Chapter 5, the frequency of 17p allele loss in sporadic carcinomas is also similar to reported series. 17p allele losses

are far less frequent in both sporadic and familial adenomas than in carcinomas, suggesting two populations of tumours as regards 17p allele loss (FAP adenoma vs. sporadic carcinoma, $X^2=23.4$, $p<0.001$; sporadic adenoma vs. carcinoma , $X^2=4.519$, $p<0.05$). The numbers are too small for comparison of YNZ22 loss between FAP and sporadic adenomas.

Table 6.3. Frequency of demonstration of 17p allele loss as detected by probe YNZ22 in FAP and sporadic neoplasms. n=number of tumours studied. Number exhibiting allele loss over number informative with percentage in parentheses.

Probe	Tumours informative		
	FAP adenoma (n=28)	Sporadic adenoma (n=8)	Sporadic carcinoma (n=41)
YNZ22	0(0%)	1(13%)	25(61%)

The only sporadic adenoma which exhibited YNZ22 allele loss (T170A) was a tubulovillous adenoma 2.0cm in diameter which had not lost any 5q markers. Clearly, little can be inferred from this data but it does suggest that there is no strong association between 17p and *APC* loss.

Comparison of histology, ploidy and allele loss data for each sporadic adenoma is shown in Table 6.4. Flow cytometric data are incomplete due to loss of stored tumour material after a freezer thaw. All sporadic adenomas examined were diploid and it is notable that the adenoma which exhibited YN5.48 allele loss was a diploid tumour, supporting the notion that *APC* inactivation is an early event. There was no particular predilection for allele loss to occur in any particular size or histological type of adenoma.

Table 6.4 Ploidy level, histological classification, size and allele loss status at 5q (*APC*) and 17p (p53) of all 20 sporadic adenomas studied. NI = non-informative, - = no allele loss, + = allele loss.

Adenoma	Ploidy level	Histology	Size	Allele loss status	
				Ch.5(*APC*)	17p(p53)
T84A	-	Tubular	0.75cm	-	NI
T87A	-	Tubular	1.0cm	-	-
T94A	-	Tubulovillous	0.5cm	NI	Insuff.DNA
T105A	-	Tubular	1.0cm	-	NI
T131A	-	Tubular	0.5cm	+	-
T140A	-	Tubulovillous	1.8cm	-	NI
T141A	-	Tubulovillous	0.5cm	-	-
T150A	-	Tubulovillous	2.8cm	+	NI
T170A	-	Tubulovillous	2.0cm	-	+
T180A	1.0	Tubulovillous	2.0cm	-	-
T182A	-	Tubular	0.5cm	NI	NI
T191A1	1.0	Tubulovillous	4.0cm	-	-
T191A2	-	Tubulovillous	0.6cm	-	-
T192A1	-	Tubulovillous	1.8cm	-	-
T192A2	-	Tubulovillous	0.7cm	-	Insuff.DNA
T237A1	1.0	Tubulovillous	5.0cm	-	NI
T237A2	1.0	Tubulovillous	5.5cm	-	NI
T237A3	1.0	Tubulovillous	1.0cm	+	NI
T237A4	-	Tubulovillous	2.0cm	-	NI
T237A6	1.0	Tubular	3.5cm	-	NI

6.4 Discussion

This chapter describes a stringent assessment of the deletion status of the *APC* gene in familial and sporadic colorectal adenomas. Multiple polymorphic DNA markers mapping very close to, and on both sides of, the *APC* gene have been analysed in each category of neoplasm. The series of experiments presented in this chapter and in Chapter 5 have been able to demonstrate clear differences in the frequency of *APC*-related allele loss in FAP adenomas, sporadic adenomas and sporadic carcinomas. These are the first data which show that the three classes of tumour represent different populations with regard to their *APC* deletion status. The results demonstrate that the development of colorectal adenomas in Familial Adenomatous Polyposis does not require a 'second hit' at the *APC* locus in addition to the inherited *APC* mutation, even though the tumours studied exhibited signs of histological aggression and aneuploidy.

Allele losses in carcinomas developing in patients with FAP have been demonstrated with markers which map to loci some considerable distance from APC (Miyaki 1990a and b, Sasaki 1989). Such deletions would certainly have been detected had they already been present in the aneuploid FAP adenomas analysed in

these experiments by use of flanking markers which span APC by as little as 2-3mb in some cases (see Chapter 4). There are published reports showing 5q allele loss in a small minority of FAP adenomas (Rees 1989, Sasaki 1989). However, these studies did not employ markers closely flanking apc. Hence it is possible that the observed allele losses in those studies were non-specific and resulted from generalised chromosomal instability. Such instability has been described, even in extra-colonic tissues in FAP (Delhanty 1983, Harnden 1984). A further explanation for the detection of allele losses detected in adenomas using the micro-satellite probe λMS8 (Rees 1989) may be due to inherent instability at the locus recognised by that probe (Wayne 1990, Wong 1987). However, another more attractive explanation exists which suggests that adenomas (familial or sporadic) which are destined to become carcinomas lose the remaining functional APC gene at an early stage. This hypothesis is supported by the data presented here and elsewhere that allele losses are detected throughout the size range of sporadic adenomas (Vogelstein 1988) and familial adenomas (Rees 1989), including those <1cm. Progression to malignancy for any individual FAP adenoma is an infrequent event when the vast number present in the colorectum of an affected patient is considered (Bussey 1975). Hence, the frequency of carcinomatous change in FAP adenomas must be substantially lower than is the case for sporadic adenomas (Morson 1983, Muto 1975) and so it is not surprising that these experiments have shown a significantly lower frequency of allele loss in FAP adenomas compared to their sporadic counterparts.

Since these data suggest that, as regards *APC* deletion, every colorectal mucosal cell has the wherewithall to become adenomatous, a hypothesis must be proposed to explain why the *whole* of the colorectal mucosa is not adenomatous in patients with FAP. Bodmer has suggested that there is natural variation in the level of *APC* gene product which rarely if ever goes below a threshold level in the normal homozygote (Bodmer 1987). However, in the deficient heterozygote (either due to an inherited constitutional mutation in FAP or to an acquired mutation in one or more colonic epithelial cells in the sporadic case) the level of *APC* gene product would drop below the threshold and the adenomatous polyp could then develop. In the deficient homozygote the APC gene product would be virtually absent and presumably would then allow carcinomatous change to progress.

One criticism of this study might be that sporadic adenomas were collected from patients who almost all had a co-existing carcinoma and the remainder were symptomatic and difficult to remove by endoscopic polypectomy. However, most published series have analysed adenomas collected from such patients and there are practical difficulties in obtaining large numbers of adenomas not associated with carcinoma, such as those obtained at colonoscopy. It will be of interest to ascertain

the true frequency of allele losses in adenomas from patients who do not also have a carcinoma.

Allele losses demonstrated in this study could represent first, or a second inactivating events at *APC*. If the deletions demonstrated in sporadic adenomas were due to a first event and a subsequent similar somatic deletion were required to allow progression to malignancy, it seems highly improbable that homozygous deletions of any marker mapping very close to *APC* was not detected in any of the carcinomas analysed in Chapter 5. The most compelling evidence that the deletions detected in these sporadic adenomas and in the sporadic carcinomas presented in Chapter 5 represent second events is the finding that in carcinomatous tissue arising in a number of patients with FAP, allele loss was detected at a marker locus some distance from *APC* and it was the allele inherited in association with the mutant *APC* allele which was retained (Miyaki 1990a and b).

Although these data, combined with published reports, suggest that the deletions observed in sporadic adenomas represent second events, it is possible that a substantial deletion could be a first event with subsequent homozygous deletion occurring due to a microdeletion or point mutation. This could occur if there is selection against two substantial losses from the region around *APC*. A colorectal mucosal cell with sufficient molecular lesions to allow adenomatous change may not be able to sustain homozygous loss of a large portion of chromosome 5 due to the presence of other important genes which are essential for cell survival. The observed allele losses could be all that is required genotypically while epigenetic inactivation of the other *APC* allele might occur by genomic imprinting (see Chapter 4). It has been suggested, with some circumstantial supporting evidence, that the 'second hit' never happens and that repression of the remaining allele by imprinting modification is retained into adulthood in some cells (Reik 1989, Sapienza 1989). There are no experimental data on imprinting in colorectal cancer but the hypothesis will be testable by search for preferential loss of one or other parental allele using the markers described here. There are no hard data as yet as to whether one or two events are required at *APC* for carcinomatous change or what types of lesion are allowable. It is also unknown whether a carcinoma can arise from a cell population which has two normal *APC* alleles. Once the *APC* gene has been cloned it will be possible to provide definitive data on this by sequencing both alleles.

Vogelstein has suggested that the role of *APC* is as a negative regulator of cellular proliferation in the colorectal mucosa and that inactivation of one copy reduces normal gene expression, allowing epithelial hyperproliferation (Vogelstein 1988). He proposes that loss of at least one allele would be required but loss of both

alleles would not be necessary. Subsequent events leading to adenomatous, then carcinomatous change might be Ki*ras* mutation (Bos 1987, Forrester 1987) or loss of tumour suppressor gene function at other chromosomal locations (Fearon 1990b), such as the gene for p53 (Baker 1989) or the DCC gene on 18q (Fearon 1990a). The findings of a high frequency of 5q allele loss in FAP carcinomas (Miyaki 1990a and b, Sasaki 1989) argue against Vogelstein's original notion that one lesion on *APC* is sufficient but all published data do support his proposals of the later events in colorectal carcinogenesis involving on 17p, 18q and Ki*ras*.

Data presented in this chapter tend to support the proposal that *APC* inactivation occurs early in colorectal carcinogenesis since there appears to be no association in adenomas of pathological and flow cytometric findings. 5q 17p allele loss data in adenomas suggest that loss of p53 is not necessarily required in association with *APC* gene loss.

This chapter has allowed some insight into the requirements for *APC* gene loss in colorectal tumours and sheds some light on the timing of such deletion in relation to the adenoma-carcinoma sequence. The definitive answer to the question of *APC* inactivation by one or two events, by point mutation or deletion, and its effect on cellular function in the colorectal mucosa will have to wait until cloning, sequencing and full characterisation of the APC gene itself.

CHAPTER 7

PULSE FIELD GEL ELECTROPHORESIS.

7.1 Introduction

Mapping data generated by linkage analysis in FAP families presented in Chapter 4 and deletion analysis in sporadic colorectal carcinomas described in Chapter 5 strongly suggest that the locus recognised by the marker EF544 lies very close to the *APC* gene itself. This chapter describes an analysis of large DNA fragments designed to detect constitutional rearrangements at the EF544 locus due to deletions involving *APC* which would be causative of the FAP syndrome in that individual patient.

Patients who develop FAP due to new mutation of *APC* but in whom cytogenetic analysis of peripheral blood leukocytes is normal must carry relatively small deletions, insertions or point mutations within *APC* resulting in the clinical manifestations of the syndrome. A difference at the molecular genetic level must exist between normal parents and affected offspring. Comparison of the fragments bearing the marker locus shown by mapping analysis to be closest to *APC* might reveal rearrangements which would be important confirmation of the proximity of that marker. It would also be an invaluable tool in targeting a specific region with a view to isolation and cloning of *APC* itself.

7.2 Methods

7.2.1 Clinical material.

In two separate families (KMD 10 and 11), I have screened the parents and siblings of the probands to be clear of FAP. Therefore both probands are new *APC* mutations. Since both have two surviving parents, these patients are suitable for pulse field gel electrophoresis (PFGE) analysis.

7.2.2 Analysis by pulse field gel electrophoresis.

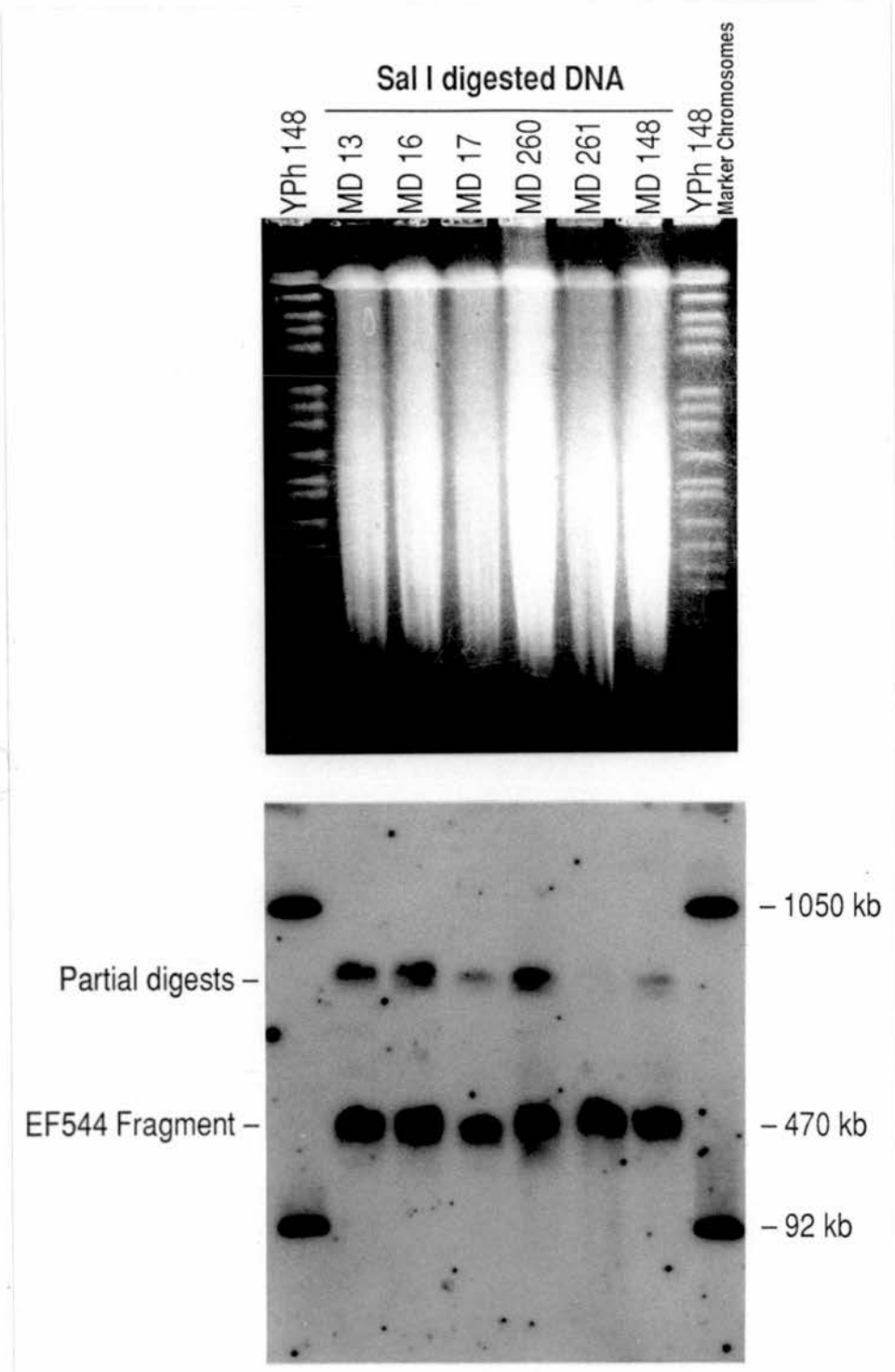
Protocols for PFGE are detailed in Chapter 2. For these experiments fresh peripheral blood leukocytes (proband and parents of KMD 10) or EBV transformed lymphoblastoid cell lines (proband and parents of KMD 11) were embedded in agarose plugs as described in Chapter 2. Plugs were digested with 4 rare cutting restriction endonucleases (Nru1, Mlu1, Sal1, Not1) and the resultant fragments were

fractionated on a pulse field gel apparatus. Marker chromosomes were those of Saccharomyces Cerevisiae (strain YPh148) which gives band sizes as shown in Chapter 2. PFGE was carried out at 100V with 200s pulse time for 5 days. Prolonged Southern blotting was carried out and filters hybridised with probe inserts of EF5.44 and separately with L5.62 as previously described.

7.3 Results

These results effectively exclude deletions involving *APC* for a distance of up to 800kb either side of EF5.44 in the two patients analysed. Figure 7.1 shows an example PFGE experiment with plugs digested with Sal1. The ethidium bromide stained gel photograph is also shown with S. Cerevisiae marker chromosomes. The autoradiograph (below) shows cross-hybridisation of a small amount of labelled contaminating pUC18 plasmid vector which carried the EF5.44 clone, despite attempts to remove all vector. This cross-reaction is of value because it reveals the relative positions of the 1050kb and 92kb marker yeast chromosome. The EF5.44 locus is included on a 470kb Sal1 fragment. No rearrangement is apparent and this was the case for all 4 enzymes with EF5.44 hybridising to a 1.2mB Nru1 fragment, a 120kb Not1 fragment and a 800kb Mlu1 fragment.

Figure 7.1 PFGE experiment analysing Sal1 EF5.44 fragments in two new *APC* mutations with both sets of parents as controls. Ethidium bromide stained gel above showing relative positions of YPh148 marker chromosomes.



7.4 Discussion

This chapter describes an unsuccessful attempt to identify constitutional rearrangements involving EF5.44 which would have been an important tool for mapping the *APC* gene. However, these experiments have excluded *APC* deletions as the cause of the FAP syndrome in these two individual patients and are compatible with the view that deletions involving *APC* are rare. This is supported by reports showing that cytogenetically detectable 5q deletions causing FAP are extremely rare (Herrera 1986, Hockey 1989, Endo 1987). It seems likely that the causative *APC* lesion in most patients may involve a point mutation. However, small deletions cannot be wholly excluded as they may been missed due to the limitations of resolution achievable by the experimental technique or because such deletions did not overlap the fragment carrying the sequences recognised by EF5.44.

The potential of this series of experiments for isolation of *APC* has not been fully realised since neither patient had a deletion. However, exclusion of deletions involving many kilobases of chromosome 5 DNA in these patients has been an important first step in the characterisation of common mutations resulting in the FAP syndrome. Identification and sequencing of the *APC* gene may well be achieved quite soon and characterisation of different *APC* mutations will then be an important part of that work.

CHAPTER 8

PRESYMPTOMATIC DIAGNOSIS OF FAMILIAL ADENOMATOUS POLYPOSIS BY DNA PROBE ANALYSIS.

8.1 Introduction

The emphasis of the data from FAP families presented in earlier chapters of this thesis has been on linkage analysis for the purposes of mapping marker probes relative to *APC*. Having established a map of the region around *APC* from linkage and deletion studies, this can be combined with published linkage analyses and a physical map of the region (Nakamura personal communication 1990). Such data can be utilised for presymptomatic diagnosis of FAP in at-risk family members in order to influence clinical management.

This chapter presents the first clinical application of DNA analysis in FAP. A prospective evaluation of the clinical value and validity of such analysis for 41 individuals within the families studied in this project in whom FAP could not be excluded due to young age or because screening had not been instigated or completed.

8.2 Patients and Methods

In brief, this analysis consists of an assessment of clinical risk derived from age at last negative colonoscopy combined with risk derived from linked DNA markers, followed by a comparison of clinical data obtained during the round of screening described in Chapter 3. This allows an assessment of the clinical value of genotype data and of the validity of the risk assigned for each individual.

Forty one members of 7 of the families (KMD1,2,3,4,6,8,14) were identified as being at risk of FAP prior to screening. Eleven (27%) had previously undergone examination of the colon and had been thought to be unaffected. There is a 50% prior risk that offspring of a patient with FAP will carry the gene defect but this risk reduces with increasing age at each negative colonic examination. Valid estimation of such age-dependent risk can only be made from age-of-onset curves derived from a population of patients who have previously been screened negative, but subsequently develop polyps. The liability classes delineating age-dependent penetrance used for this study are shown in Table 8.1 and are derived from

published age-of-onset curves (Murday 1989). The prior risk for each individual screened is shown in Table 8.2 and was assigned as 0.5 for those who had never been screened. Risk was assigned for those who had previously been examined as <0.5 depending on the age at last negative screen. For example the prior risk for an individual last screened negative at age 25 was 0.10, or a 10% chance of being affected.

Table 8.1 Liability classes and age-dependent penetrance in FAP (from Murday 1989).

Liability class	Age (years)	Penetrance
1	0-9	0.0001
2	10-14	0.5
3	15-19	0.75
4	20-24	0.85
5	25-29	0.90
6	30-34	0.99
7	35+	0.999

Genotypes were obtained for each individual and all members of that family using 6 DNA probes as described in Chapter 4. The final linkage map presented in Chapter 4 includes data from a number of the individuals who underwent screening. A preliminary run with the LINKAGE program prior to any screening revealed substantially the same mapping data, though the lod-scores were lower (data not shown). Hence, for the purposes of this analysis, probe order and recombination fractions for linkage to *APC* were derived by combining the preliminary linkage analysis with a variety of independent data including published linkage analyses (Bodmer 1987, Leppert 1987, Meera-Khan 1988, Nakamura 1988, Varesco 1989, Tops 1989), *in situ* hybridisation analysis (Varesco 1989), physical mapping data (Nakamura personal communication 1990) and the deletion analysis in sporadic colorectal carcinomas presented in Chapter 5 (Ashton-Rickardt 1989). The amalgamated data confidently support a locus order of pi227-C11P11-ECB27-L5.62-APC-EF5.44-YN5.48 with recombination fractions of 0.03, 0.03, 0.02, 0.02, 0.01, 0.02 in respective intervals. The 95% probability limits for linkage of probes pi227, C11P11, L5.62 and YN5.48 to *APC* are narrow and so have not been taken into account in risk analysis. In addition, probability limits have little influence on risk estimation when close flanking markers are informative and when independent clinical and genetic risk estimations are combined, as reported here.

Risk estimations for genotype data were calculated using multipoint linkage analysis with the MLINK program in the LINKAGE 5.02 group of programs on an

IBM Series 70 80386 computer with math coprocessor. Computation times for KMD 1 took around 4 hours due to the number of unknown genotypes in early generations.

A preliminary risk estimation was calculated for each individual by combining genotype data and prior risk. All at-risk family members over 13 years of age were then invited for screening of the large bowel by colonoscopy or by rigid sigmoidoscopy and barium enema. All polyps were biopsied for histological confirmation. In addition to colonic screening, affectation status was also assigned according to the presence or absence of congenital hypertrophy of the retinal pigment epithelium (CHRPE) (Blair 1982, Chapman 1989, Iwama 1990, Polkinghorne 1990, Traboulsi 1987). Individuals with more than three lesions, and those with diagnostic large and/or bilateral lesions were considered to be gene carriers while those with less than 3 lesions were considered negative as suggested by Chapman (Chapman 1989). Retinal assessment by direct and indirect ophthalmoscopy was carried out by myself initially and then independently by an ophthalmologist (Professor CI Phillips) and a fundal camera operator (Mr S Gairns). There were no inconsistencies in the assessment of numbers, laterality and size of the retinal pigmentation. All CHRPE lesions were photographed and examples are shown in Chapter 3.

Empirical assignment of a penetrance value for CHRPE was required as there are conflicting published data. One study has suggested that expression of CHRPE tends to co-segregate faithfully with colonic manifestations of the gene defect (Chapman 1989). However, other reports suggest around 90% of affected individuals in any given FAP family exhibit CHRPE (Iwama 1990, Polkinghorne 1990, Traboulsi 1987). Therefore, penetrance has been taken as 0.90 in order to minimise any possibility of false reassurance to patients. Family members were also assessed clinically and radiologically for the presence of cranio-facial osteomas. Orthopantomography was not carried out in children. Two children (KMD8/4-6 and 4-7) exhibited gross cranial osteomas and so have been assigned as gene carriers. Unfortunately, it was not possible to carry out direct or indirect ophthalmoscopy in these patients due to practical considerations.

Clinical screening by any modality was completed for 34 individuals (83%); 18 (43%) by colonic examination, 24 (59%) by ophthalmoscopy and 9 (21%) assessment for cranio-facial osteomas (see Table 8.2). The final clinical diagnosis based on colonic, retinal or bony findings for those who are affected is a hard end-point against which to evaluate the genotype-derived risk estimation. However for those who appeared to be unaffected there is no such 'gold standard'. Therefore, following a negative screen, the new clinical data were added in to a further

computer calculation in order to reassign the risk estimation to produce a residual (final) risk. All relatives of these individuals who were shown to be affected by the current round of screening were included in this calculation, thereby improving linkage phase information. Residual risk in some cases was calculated as less than that of the general population, thereby effectively excluding FAP and serving as a hard end-point. In others the residual risk was sufficiently low to influence screening policy for their future care.

8.3 Results

Table 8.2 shows the prior risk, probe derived risk, combined probe/prior risk with residual risk for those screened negative on the current round of examinations and all references in the text below are to these data. Risk estimation derived from genotype data has been assigned for the majority of the study population. One or more probes were informative in all but two cases. The frequency of probe informativity ranked in descending order was pi227(83%; 95% confidence intervals (CI), 71.2-94.7%), YN5.48(51%; 95% CI, 35.6-66.8%), L5.62(46%; 95% CI, 30.8-61.9%), C11P11(41%; 95% CI, 26.1-56.9%), EF5.44(24%; 95% CI, 11.0-37.8%), ECB27(2%; 95% CI, 0-7.3%). Data from informative flanking probes were obtained for 28 of the 41 individuals studied (68%; 95% CI, 53.8-82.8%). The use of pi227 and YN5.48 in combination was particularly efficient since at least one probe was informative in 35 instances (85%) and both probes were informative in 21 (51%) out of all 41 individuals tested.

Table 8.2 At-risk family member, prior risk, probe derived risk and DNA probes informative (P=pi227, C=C11P11, EC=ECB27, L=L5.62, EF=EF5.44, Y=YN5.48). Combined probe and prior risk is only indicated if prior risk was <0.5, otherwise combined risk is as for probe derived risk alone. Screening modality: 1=colonoscopy and biopsy, 2=CHRPE phenotype, 3=clinical and/or radiological osteomas. Final clinical diagnosis is shown with residual risk in parentheses. ? indicates unknown final clinical status (ie young child with unknown CHRPE status). Broken lines separate individuals from different families. The residual risk for individuals 4/4-1 and 4/4-2 was influenced when their father 4/3-6 was screened and shown to be affected.

At-risk individual	Prior risk	Any probe informative	Probes informative	Probe derived risk	Combined probe and prior risk	Method of screening	Clinical status (Residual risk)
1/4-25	0.50	Y	P,C,Y	0.0040	-	1,3	-ve(<0.000007)
1/5-7	0.25	Y	P,C,EC,L,EF	0.9503	0.8636	1,2,3	affected
1/5-13	0.15	Y	P,Y	0.0032	0.00056	1,3	-ve(0.000003)
1/5-14	0.10	Y	P,Y	0.0032	0.00036	1,3	-ve(0.000003)
1/5-15	0.25	Y	P,L,Y	0.0006	0.0002	1,3	-ve(0.000006)
1/5-16	0.10	Y	P,Y	0.0032	0.00036	1,3	-ve(0.000036)
1/5-17	0.50	Y	P,Y	0.9968	-	1,2,3	affected
1/5-18	0.50	Y	Y	0.0865	-	(declined)	?(0.0865)
1/5-19	0.25	Y	P,C	0.0680	0.0237	1	-ve(0.0179)
11/5-20	0.25	Y	P,C	0.0680	0.0237	1	-ve(0.0179)
1/6-1	0.50	Y	P,L	0.0200	-	2	-ve(0.0011)
1/6-2	0.50	Y	P,L,EF	0.9998	-	2	affected
1/6-3	0.50	Y	P,L	0.9800	-	2	affected
1/6-4	0.50	Y	P,L,EF	0.9998	-	2	affected
1/6-7	0.50	Y	C,L,EF	0.0004	-	2	-ve(0.00002)
1/6-8	0.50	Y	C,L	0.0200	-	2	-ve(0.0011)
1/6-9	0.50	Y	C,L	0.0200	-	2	-ve(0.0011)
1/6-10	0.50	Y	C,L	0.9799	-	2	affected
2/2-4	0.10	Y	P,C,EF,Y	0.9991	0.9920	1,2	affected
2/2-6	0.15	Y	P,C,EF,Y	0.8777	0.5588	1,2	affected
2/2-7	0.25	Y	P,C,EF,Y	0.0007	0.0002	1,2	-ve(0.000017)
2/3-1	0.50	Y	P,C,Y	0.9798	-	1,2	affected
2/3-2	0.50	Y	P,Y	0.0026	-	1,2	-ve(0.00006)
3/3-1	0.50	Y	P,Y	0.9612	-	2	affected
3/3-2	0.50	Y	P,Y	0.9272	-	2	affected
4/3-6	0.50	N	-	-	-	1	affected
4/4-1	0.50	Y	P	0.4378	-	(not performed)	?(0.8757)
4/4-2	0.50	Y	P	0.1561	-	(not performed)	?(0.3122)
6/4-1	0.50	Y	P	0.0952	-	2	affected
6/4-2	0.50	Y	P,Y	0.7728	-	2	-ve(0.1518)
6/4-3	0.50	Y	P,Y	0.0032	-	2	-ve(0.0032)
8/4-1	0.50	Y	P,C,L,Y	0.00065	-	1,2	-ve(0.000018)
8/4-2	0.50	Y	P,C,L,Y	0.00065	-	1,2	-ve(0.000036)
8/4-4	0.25	Y	P,L	0.9128	0.7772	1,2	affected
8/4-5	0.50	Y	P,L,EF	0.9998	-	3	affected
8/4-6	0.50	Y	P,L,EF	0.9998	-	3	affected
8/4-7	0.50	Y	P,L,EF	0.9998	-	(not performed)	?(0.9998)
8/4-10	0.50	Y	P,C,L,Y	0.9994	-	(not performed)	?(0.9994)
8/4-11	0.50	Y	P,C,L,Y	0.5960	-	2	-ve(0.1408)
8/4-12	0.50	Y	P,C,L,Y	0.9994	-	(not performed)	?(0.9994)
14/5-1	0.50	N	-	-	-	(not performed)	?(0.5)

Figure 8.1 shows an example family (KMD 2) demonstrating segregation of the chromosome carrying the mutant *APC* gene (d) and haplotypes for all informative probes. Haplotype data of this kind are extremely powerful since recombination events and errors of genotyping can be easily identified. The haplotypes of the chromosome harbouring the mutant *APC* gene and that with the normal gene can be followed through the family to predict risk as described below. Figure 8.1 consists of the provisional pedigree for KMD 2 prior to the screening described in Chapter 3 (for final version see Appendix A and Figure 4.2). Half-filled symbols indicate those who were at-risk at the outset of this project. Two individuals in F2 (KMD 2/2-4 and KMD 2/2-6) and 1 in F3 (KMD 2/3-1) with shaded haplotypes were at high risk of carrying the disease gene and the calculated risk is shown in Table 8.2. Those screened negative are shown with an unshaded haplotype (KMD 2/2-7 and KMD 2/3-2) and the liability class following screening is shown in each case. On the initial calculation, a risk of 0.88 was assigned to KMD 2/2-6 since she inherited the chromosome with a recombination event between C11P11 and EF5.44 (see Table 8.2). This risk is calculated because EF5.44 predicts an affected status while C11P11 predicts an unaffected status. Therefore, there has been a recombination event which could have occurred at any point in the interval EF5.44-C11P11 and the computer analysis takes this, and the respective recombination fractions, into account when assigning a risk estimation. This case serves to stress the value of data from.flanking markers because if only C11P11 had been informative, the risk for that patient would have been calculated as only 0.07.

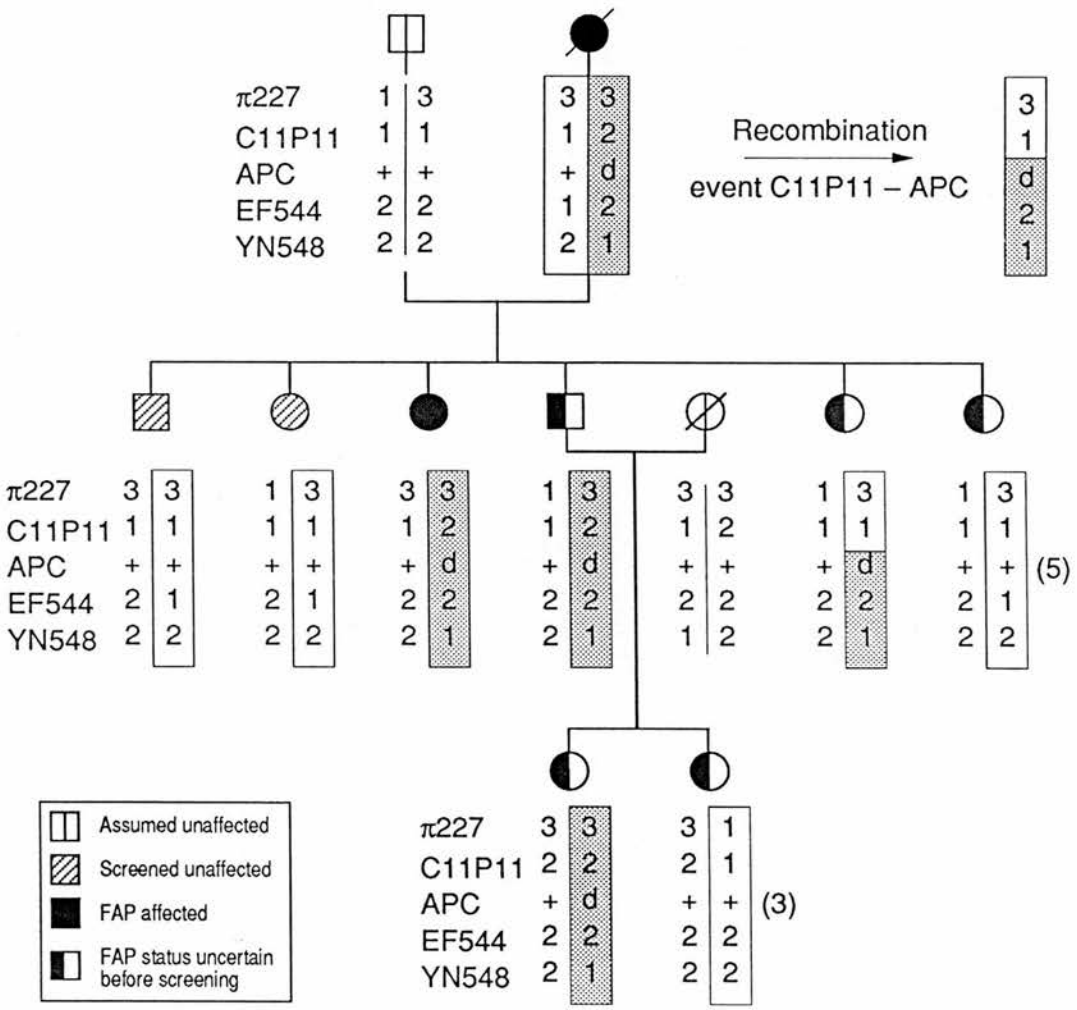
Fourteen of the 15 patients screened and shown to be affected (93%) had been assigned probe derived risks of 0.93 or greater. Previously, 4 of these individuals (KMD 1/5-7, KMD 2/2-4, KMD 2/2-6, KMD 8/4-4) had been discharged from follow-up on the basis of negative colonic screening. All 4 individuals are actually affected and KMD 1/5-7 already had carcinoma *in situ* in the resected colon (see Chapter 3). These patients may well have developed infiltrative colorectal cancer had they not been part of this study. The level of combined risk in these cases is lower than the probe derived risk alone because of the earlier negative screening. These cases emphasise the importance of DNA analysis in minimising inadequacies in clinical screening.

An initial combined risk of 0.095 was assigned for one individual (KMD 6/4-1) which was at variance with clinical findings that indicate he is affected. However, only one probe was informative in this case, resulting in a level of risk which would not have influenced clinical management.

Eighteen individuals were screened negative on the current round of examinations. Fourteen of these (78%) had been assigned probe derived risk

estimations of around 0.01 or less and 16 (89%) assigned a risk of <0.10. Combining probe information and clinical data based on the negative findings at this examination, residual risk estimations of 0.001 or less were assigned to 16/18 (89%). Ten individuals (56%) had residual risks substantially less than 0.0001, which is the incidence rate of FAP in the general population.

Figure 8.1 KMD 2 showing segregation of the shaded haplotype with the disease (d) allele in three generations. The haplotype for the deceased affected individual in F1 has been inferred. The unshaded chromosome is inherited with the normal allele (+). The clinical liability class for those screened negative during the current round of examinations is shown in parentheses next to the + allele. There has been a recombination event in the interval C11P11-*APC*, resulting in the haplotype **31d21** in of the offspring in F2.



8.4 Discussion

The aim of screening the colon of patients with FAP is to identify gene carriers who require prophylactic colectomy. However, there is a substantial burden of screening procedures undergone unnecessarily by the 50% of at-risk individuals who are actually unaffected. In addition, young adults at risk and affected parents who wish to know whether or not they have passed on the mutant gene to their offspring suffer psychological morbidity from the uncertainty inherent in the prolonged clinical screening required.

This study highlights the practical clinical value of risk analysis using linked DNA probes. The validity of probe derived risk estimation has been demonstrated and genotype information from a simple blood test, when combined with independent clinical data, provides an estimation of residual risk for each patient which can be used to influence future screening policy. The use of linked DNA markers can avoid inappropriate discharge of gene carriers after sub-optimal or foreshortened screening protocols. In this study population, 4 individuals aged between 30 and 40 years would almost certainly have developed colorectal cancer had tracing, genotyping, clinical screening and subsequent surgery not been undertaken. This serves to emphasise the need for continued colorectal surveillance until middle age in patients assigned high risk estimations or those with indeterminate risk.

A clear distinction must be made between what is an acceptable level of risk to inform a patient of the likelihood that he/she is affected and what is an acceptable level of risk to allow a reduction in screening. While an initial combined risk of 0.7 would allow the clinician to advise a patient that it is probable he/she is affected, a probe derived risk of 0.01 or less seems the necessary level required before any radical change in screening policy can be undertaken. The rationale for selecting this level of risk is that an at-risk individual screened negative for polyps and for CHRPE on first examination at the age of 15 who has a probe derived risk of 0.01 has a 3 in 10,000 chance of being affected. At this level it would be reasonable to advise only a single further colonic examination at the age of 30 years when the residual risk will be only 0.00001 if he/she is again screened negative, ten times less than the incidence of the disease in the general population. For individuals aged 15 with no polyps and negative CHRPE who have a probe derived risk of 0.1-0.01, it would also be reasonable to reduce colonic screening frequency to perhaps once every 2-3 years since the residual risk is only 0.004.

The methodology employed here merits some discussion. Locus order was based on an amalgamation of independent data from linkage analysis and data from

rare recombination events described in Chapter 4, published rare recombination events (Tops 1989), published linkage data (Bodmer 1987, Leppert 1987, Meera-Khan 1988, Nakamura 1988, Varesco 1989, Tops 1989), deletion analysis in colorectal cancers (Ashton-Rickardt/Chapter 5) and physical mapping data (Nakamura personal communication 1990). The recombination fractions between marker and *APC* are somewhat greater than might be suggested by summation of published data but it is important not to place a marker over-optimistically close to *APC* and give ill-informed reassurance to patients. Liability classes have been assigned based on the age-of-onset curves of Murday (Murday 1989) since it is the risk of developing polyps after being screened negative which is of importance to risk analysis. These penetrance values are substantially different from those suggested by curves derived from age of *presentation* of polyps which are the data usually quoted (Muto 1975).

Flanking markers were informative in 68% of individuals tested with a 95% confidence interval of 53.8-82.8%. Risk estimation is extremely accurate when flanking markers are informative and the risk assessments derived from each probe concur. In practice the phenomenon of genetic interference (where there is a selection against the occurrence of two recombinations close to each other) ensures an even greater diagnostic accuracy than that suggested by the computer calculated risk shown here. Recombinations between flanking markers were identified in four individuals (KMD 2/2-6, KMD 6/4-2, KMD 8/4-4, KMD 8/4-11; see Table 8.2). The value of informative flanking markers cannot be over-stressed since such recombination events can be recognised and taken into account in the risk calculation. If only a single marker is informative then, in a proportion of patients (dependent on recombination fraction), crossovers which occur between that marker and APC will not be recognised.

At-risk family members and their parents universally welcomed a simple blood test with the potential to give an estimation of the risk of having inherited the mutant gene. It was clear that prolonged and repeated examination of the colorectum was a burden to all those who required it. Indeed one of the study group (KMD 1/5-18) agreed willingly to the blood test but refused to have any colonic or ophthalmological examination despite counselling. Fortunately, his genotype suggests he is unaffected although he and his children will be included in FAP screening programs with regular invitations for endoscopic follow-up.

There were no affected mothers or mothers-to-be who would have considered prenatal diagnosis with a view to termination of a pregnancy. One perinatal diagnosis (K8/4-12) was carried out at the parents request by genotyping DNA purified from placental tissue. Maternal contamination of the DNA could not

influence this analysis since the mother was homozygous for flanking markers and she did not carry the marker allele which cosegregated with *APC* in her affected husbands family. The parents were counselled prior to the test and were fully aware of the implications of a test at such a young age. Unfortunately the child is almost certainly affected but the parents are glad to know this and felt they would have suffered from the uncertainty otherwise.

The data presented here demonstrate that linked DNA probe markers can now be used for preclinical risk estimations in FAP. Genotype data can be incorporated into current screening protocols to reduce the burden of colonic screening and to minimise the risk of gene carriers being lost to follow-up. An integrated approach to screening individuals at risk of FAP can now be instigated, involving genotyping, retinal examination and colonic screening in suitable families. Linked DNA markers will continue to have a central role in presymptomatic diagnosis of FAP even after identification and cloning of the *APC* gene with subsequent characterisation of common mutations within it. Families which do not carry easily typed mutations will require DNA probe derived risk estimations and linked markers will also be needed for confirmation of mutation analysis.

There are substantial numbers of individuals who could benefit from preclinical risk estimation using linked DNA probes. If the birth rate in the United Kingdom is taken as approximately 700,000 per annum and the incidence rate of FAP as 1 in 8,000 live births, then there are around 2600 affected individuals currently under 30 years of age and a further 90 are born each year. However, since new *APC* mutations make up 30% of all cases and such individuals are not suitable for preclinical diagnosis using linked DNA markers, these figures have to be modified accordingly. In addition, FAP is an autosomal dominant disorder and so affected parents have roughly equivalent numbers of affected and unaffected offspring, but all of whom require screening. Therefore, one can calculate that there are currently 3700 at-risk individuals in the United Kingdom who would benefit from the DNA analysis presented in this chapter with a further 126 born annually.

CHAPTER 9

DISCUSSION

The work presented in this thesis has focussed on the involvement of *APC* aberrations in causation of the clinical manifestations of the FAP syndrome and on the putative role of *APC* as a tumour suppressor gene in colorectal carcinogenesis. A high resolution genetic linkage map including 6 RFLP markers located around *APC* has been generated. This has been used to interpret allele loss data from FAP adenomas and from sporadic adenomas and carcinomas. The work has demonstrated a high incidence of 5q allele losses in sporadic carcinomas and shown the specificity of such loss of genetic material for the *APC* gene. A lower incidence in sporadic adenomas has been demonstrated and the relationship of these findings to the situation have been discussed. Data have been generated which are of direct clinical relevance to patients at-risk of FAP and which can radically alter current follow-up protocols. Clinical and molecular genetic findings have combined to constitute powerful evidence to suggest an important role for *APC* aberrations in colorectal carcinogenesis.

Research into the molecular genetics of colorectal cancer has moved on at a breath-taking pace since this work began and the work presented here must be taken in the context of intensive efforts by several research groups working world-wide in this exciting field and the data presented here are well supported by reports published in the world literature.

Attempts to map *APC* using cytogenetic techniques became largely redundant following regional localisation of *APC* to chromosome 5q21-22 in 1987 by groups in London (Bodmer 1987) and Salt Lake City (Leppert 1987). Therefore, attention was concentrated on the ascertainment and documentation of a large collection of FAP families for genetic linkage. Cytogenetic assessment was restricted to analysis of one or more affected individuals as part of that ascertainment. Failure to demonstrate karyotypic abnormalities in any of these families is not surprising since only one further cytogenetic abnormality has been reported in two brothers (Endo 1987, Hockey 1989) after the original report of Herrera (Herrera 1986). However, karyotype analysis was an important aspect of this project since the identification of a single patient with a small deletion or, most important of all, a chromosomal translocation would have been invaluable in final localisation of *APC* itself.

9.1 Genetic linkage analysis and preclinical diagnosis of FAP.

After the establishment of primary genetic linkage (Bodmer 1987, Leppert 1987), a limited number of linkage analyses in FAP were published (Meera Khan 1988, Murday 1989, Nakamura 1988, Varesco 1989). Bodmer believed C11P11 to be very close to *APC*, but data presented in this thesis and reported elsewhere was first to identify 3 obligate C11P11-*APC* recombinants allowing ordering of C11P11 and pi227 around *APC* (Dunlop 1989a, Dunlop 1989b). A single recombination event between C11P11 and *APC* was reported by one group (Aldred 1988) but there were no other informative markers to provide useful mapping information. Ordering of *APC* relative to pi227 and YN5.48 in a linkage analysis was later established by identification of a cross-over in the interval YN5.48-*APC* which was non-recombinant for pi227-*APC* (Tops 1989).

The high resolution map presented in Chapter 4 represents the most detailed and confident linkage map of the region to date and is currently in press (Dunlop 1990). Mapping data from linkage analysis are invaluable tools for the identification and isolation of genes and the map constructed as part of this project has helped towards final isolation of *APC*. The linkage data have already been utilised by Yusuke Nakamura in constructing linkage and physical maps of the region (see below) and a number of candidate clones have now been identified.

The linkage map includes a high density of markers such that presymptomatic diagnosis of FAP by DNA analysis can now be used in the clinical setting since flanking markers have been identified and diagnostic certainty can be very high. The first direct application of recombinant DNA technology to clinical problems raised by FAP is presented in Chapter 8 and the power of such analysis is formally tested. Recommendations are made for an integrated screening policy for those at-risk of FAP. The combination of risk estimations derived from colonic screening, retinal screening and RFLP genotype can reduce the risk for those apparently unaffected when screened in their late teens to such low levels that further endoscopy of the colon can be reduced to a single examination around the age of 30 years. This is clearly of great benefit to individuals who no longer need to undergo unnecessary yearly sigmoidoscopy. In addition, missed cases of FAP presenting late with a carcinoma due to inadequate follow-up should now be avoided. A number of cases discussed in Chapter 4 exemplify the value of DNA analysis since the development of colorectal cancer was prevented despite inferior clinical follow-up. RFLP genetic linkage analysis to provide risk estimations for at-risk family members will soon be routine practice and mapping data generated in this project have contributed to the body of knowledge which will be used by clinical genetic units involved in setting up a UK diagnostic service for FAP.

9.2 Allele losses in adenomas.

Data presented in Chapter 6 constitute a stringent analysis of the deletion status of *APC* in colorectal adenomas. Chromosome 5 allele losses in FAP and sporadic adenomas have been reported by other groups (Okamoto 1988, Rees 1989, Solomon 1987, Vogelstein 1988). Allele losses at marker loci closely flanking *APC* has been assessed and, due to the density of markers analysed here and to the large numbers of FAP and non-FAP tumours examined, data presented in this thesis convincingly demonstrate that FAP adenomas, sporadic adenomas and sporadic carcinomas comprise three different tumour populations as regards *APC* deletion status. These data demonstrate that there is no requirement for two 'hits' at *APC* for the development of FAP adenomas. As discussed below, a second hit has been shown to occur in colorectal cancer developing in patients with FAP. Therefore it would appear that homozygous inactivation of *APC* does occur in colorectal cancers developing in FAP patients. The question is whether allele losses detected here in sporadic adenomas represent only a hemizygous aberration of *APC* or whether it implies a homozygous lesion with the more substantial lesion 'unmasking' a mutant *APC* allele on the remaining homologue. In Chapter 6 the available data are discussed and it is proposed that a second event at *APC* may commit the adenoma to progression along the pathway to malignant proliferation. If confirmed, this could represent a marker of a highly mutagenic large bowel content for any patient who has undergone removal of one or more adenomas with such chromosome 5 allele loss. This may suggest an increased incidence of developing further adenomas with a high risk of subsequent malignant transition in any given adenoma. Hence, subsequent patient follow-up may be influenced in order to address such increased risk of colorectal neoplasia.

9.3 Allele losses in carcinomas.

The demonstration of chromosome 5 allele losses in sporadic colorectal cancer by Solomon (Solomon 1987) suggested that *APC* was a tumour suppressor gene involved in colorectal carcinogenesis. Inactivation of both copies of *APC* would imply a recessive determinism which Knudson suggests may be a rate limiting step in colorectal carcinogenesis (Knudson 1989). The allele losses detected by Solomon involved loss of a substantial amount of genetic material from chromosome 5 and so loss of *APC* itself could only be inferred from such data. However, this indirect approach of searching for heritable cancer predisposition genes of clinical interest by looking for allelic deletions in the corresponding tumour tissue has already proven invaluable in tracking down several such genes including those involved in

retinoblastoma, von Hippel-Lindau syndrome and multiple endocrine neoplasia (MEN 1) (Ponder 1988, Wildrick 1989).

More substantial evidence was required to demonstrate that apc was indeed the 'target' tumour suppressor gene. A number of authors reported chromosome 5 allele losses in colorectal cancer tissue following Solomon's original publication (Delattre 1989, Law 1988, Okamoto 1988, Sasaki 1989, Wildrick 1988). The frequency of such losses was around 30-40% but all of these groups used marker probes which map some considerable distance from apc and so none could make specific comment on the target chromosomal region due to the small number of markers studied and to the lack of confident mapping data. However, one group were able to construct a limited deletion map of chromosome 5q (Vogelstein 1988) but again the mapping data were weak and the markers assessed for allele loss were not close to or flanking *APC*.

Work presented in this thesis combined with that of my co-workers in Edinburgh University was first to demonstrate the true frequency of allele loss in sporadic colorectal cancer. Over 50% of such tumours exhibit chromosome 5 allele losses, much higher than previously reported. This finding is due entirely to the assessment of allele loss at marker loci mapping close to, and on both sides of *APC* (Ashton-Rickardt 1989). The specificity for *APC* was also demonstrated due to the confidence of the linkage map presented in Chapter 4. These data clearly demonstrate that *APC* is indeed the target tumour suppressor gene on chromosome 5q involved in colorectal carcinogenesis.

As discussed in Chapter 5, chromosome 5 allele losses occur in carcinomas arising in patients with Familial Adenomatous Polyposis at a similar frequency to sporadic cancers and the extent of the deletion corresponds to that seen in sporadic colorectal adenomas and carcinomas (Sasaki 1989). It is the allele co-inherited with the mutant *APC* gene which is retained in the malignant tissue (Miyaki 1990a and b). This is compelling evidence supporting a tumour suppressor function for *APC*. Further supporting experimental evidence comes from application of the somatic cell hybrid techniques employed by Stanbridge for chromosome 11 (Stanbridge 1981). Recently, a single human chromosome 5 was introduced into a murine hepatoma cell line which resulted in suppression of tumorigenicity of the carcinoma cell line in nude mice (Sasazuki 1989). This indicates one or more chromosome 5 genes have tumour suppressing capabilities and by inference, implicates *APC* as a tumour suppressor gene. The final proof that *APC* is a tumour suppressor gene will require isolation and cloning of *APC* with subsequent demonstration that *APC* can prevent or reverse a tumorigenic phenotype as has been shown for Rb-1 (Huang 1988).

One important aspect of the involvement of *APC* deletions in sporadic colorectal cancer has not been addressed in this thesis, namely the prognostic value of *APC* loss. It will be of interest to compare the clinical outcome of patients with a cancer which has *APC* related allele loss with patients in whom the tumour shows no such loss. If the hypothesis (discussed in Chapters 5 and 6) is correct that *APC* inactivation is an early event and may indeed be a universal phenomenon with those tumours which do not show *APC* related allele loss having intragenic lesions, then there should be no influence on prognosis. Since a relatively small number of patients were involved in this study and the follow-up period ranged from 18-30 months, I have not pursued this aspect of *APC* influence on clinical outcome. Such a study is planned on a larger scale by combining follow-up data on my own series of patients with colorectal cancer with the Edinburgh University series. However, we plan to assess survival and recurrence data with a minimum follow-up of three years.

9.4 Heritable susceptibility to non-FAP colorectal cancer and tumour suppressor genes.

As discussed in Chapter 1, the gene for p53 and the DCC gene on chromosome 18q appear to be tumour suppressor genes involved in colorectal carcinogenesis (Baker 1989, Fearon 1990). Data presented in this thesis and recent world literature suggest a tumour suppressor function for *APC*. Several other potential tumour suppressor loci relevant to colorectal cancer have been identified by allele loss studies (Vogelstein 1989). All of these genes could represent colorectal cancer susceptibility genes. Clinical heterogeneity of age-of-onset, penetrance in dominant colon cancer families, site and tumour aggression all suggest that genetic heterogeneity may well have a substantial influence on genetic susceptibility to colorectal cancer. The scanty molecular genetic evidence to date would support this notion. Constitutional mutations in any one of the tumour suppressor genes listed above could render the colonic epithelium more susceptible to a further 'hit' at the same locus or at another tumour suppressor locus. The first hit may be sufficient to account for the widespread increase in epithelial proliferation demonstrated in the colorectum of patients with colonic adenomas and carcinomas (Lipkin 1984, Terpstra 1987). Such an event may prime the colonic epithelium for further mutagenic events perhaps by releasing some aspect of cellular growth control or contact inhibition. However, several tumour suppressor genes may require homozygous inactivating mutations and several oncogenes require activation before the establishment of a clone capable of all of the clinical manifestations of malignancy. It is also possible that one or more of such putative colorectal cancer

susceptibility genes have no direct effect on the colorectal mucosa itself but exert their effect by altering bile salt profiles or by inactivating a de-toxifying liver enzyme. Isolation and cloning of candidate tumour suppressor genes will be of great interest not only because of their involvement in the biology of colorectal cancer, but also because of a possible influence on heritable susceptibility to large bowel malignancy.

Evidence to implicate *APC* as a colorectal cancer susceptibility gene has been discussed in Chapter 1.1.7 and consist of the findings of an increased prevalence of mandibular osteomas in patients with non-FAP colorectal cancer (Sondergaard 1985), those with colorectal adenomas (Sondergaard 1986) and in the Cancer Family Syndrome (Sondergaard 1985). In addition, *APC*-linked DNA markers have exhibited linkage to a disease gene resulting in variable numbers of adenomatous colorectal polyps in one large kindred (Leppert 1990). The demonstration in this thesis of a probable tumour suppressor function for *APC* in the colorectal mucosa lends further support to the notion that *APC* is involved in colorectal cancer susceptibility. This hypothesis can now be tested by carrying out linkage analyses in non-polyposis colorectal cancer families. This will be more problematical than the similar analysis carried out in FAP families because of the high mortality rate of those affected and also because of the reduced level of penetrance. However, using intragenic probes and probes mapping very close to *APC*, it should be possible to confirm whether or not *APC* is indeed a colorectal cancer susceptibility gene in the foreseeable future.

The study of the involvement of constitutional mutations in specific tumour suppressor genes resulting in colorectal cancer susceptibility is one of the most exciting aspects of future research in human molecular genetics. One extremely important aspect of this work will be the introduction of a mutated tumour suppressor gene into a mouse germline. The resultant transgenic animal could represent a true model of human colorectal carcinogenesis which would not depend on carcinogen-induced tumours. Already a dominant mutation has been induced in the mouse which predisposes to intestinal neoplasia very similar to human colorectal cancer (Moser 1990). It will be of great interest to determine whether a murine gene homologous to one of the candidate human tumour suppressors is the gene involved in this case. A more elegant assessment of the role of *APC* in heritable colorectal cancer susceptibility will be to introduce a mutant form of *APC* into a murine germline in transgenic experiments. This work is already in its early stages in Edinburgh University Pathology Department and clearly has enormous potential to further our understanding of the biology and the heritability of colorectal cancer.

9.5 Towards isolation and cloning of *APC*.

Mapping of six polymorphic DNA markers around *APC* is a step towards eventual isolation and cloning of the gene itself. The data presented here suggest that EF5.44 is the marker locus which is closest to *APC* of those currently available. PFGE analysis presented in Chapter 7 failed to show any rearrangement of DNA fragments up to 800kb in size in the two patients studied. These data could suggest that *APC* is more than 800kb from EF5.44 but another, and perhaps more likely, explanation is that the genetic lesions in the *APC* gene in the two FAP patients studied were not detectable by analysing the large DNA fragments generated by PFGE analysis. Small deletions or insertions and point mutations would not be resolved by PFGE. Ideally I would have wished to carry out PFGE analysis using the EF5.344 probe on one affected member of every family collected with an unrelated individual as a control. However, the control data would not have been so secure as those described and used in Chapter 7. Unfortunately due to shortage of time, it was not possible to complete such a series of experiments. However, data from deletion analysis in colorectal cancers generated by my co-workers in Edinburgh University, which includes data presented in Chapter 5, do support the notion that EF5.44 is close to *APC*, since the frequency of allele loss is very high in cancers which show 5q interstitial deletion. The next step would be to assess whether the EF5.44 probe DNA fragment, or the cosmid clone it was derived from, carries sequences of transcribed gene. This could be tested by assessing whether the DNA sequence recognised by the probe is phylogenetically conserved by demonstration of probe hybridisation to DNA purified from tissues of a number of different animal species in what is known as a 'zoo blot' experiment. Another method would be to analyse the DNA sequence of the marker clone to assess whether the sequence recognised by the probe carries an open reading frame corresponding to known base triplets coding for amino-acids, suggesting the presence of a transcribed gene. Due to shortage of time on my research fellowship, I was unable to carry out these experiments but my collaborator, Dr. Y. Nakamura, has subsequently shown that the cosmid clone of EF5.44 does not carry transcribed sequences (Nakamura personal communication 1990) and hence cannot comprise exonic sequences of apc. Dr. Nakamura and his group in Tokyo are currently involved in an intensive effort to identify and clone the apc gene using the techniques described here of linkage mapping of new markers, assessing the frequency and nature of allele loss at that locus in colorectal cancer tissue, PFGE analysis of markers which seem to be close and then assessing each candidate marker for a high degree of cross-species sequence conservation and for features of a transcribed gene and in particular, whether the marker could include apc sequence itself. The amount of experimental

work required is enormous and the race to clone *APC* currently involves groups in London (Bodmer), Salt Lake City (White), Baltimore (Vogelstein) and Tokyo (Nakamura).

Attempts to identify and clone *APC* will be hampered by the absence of a biochemical phenotype characteristic of the gene defect, such as a specific enzyme abnormality, which would provide a indication as to which DNA sequences might encode for the *APC* product. Therefore, the identification of the *APC* gene relies wholly on molecular genetic techniques to identify DNA fragments which might be the gene and then to assess whether each candidate fragment in turn has features in keeping with those predicted for *APC*. The difficulties will be further compounded since constitutional apc mutations will probably be small deletions or point mutations which will be difficult to detect in a gene such as *APC* which is probably large, as suggested by a high new mutation rate for FAP (Reed 1955). Identification of a gene whose effects are the result of loss of function will be substantially more difficult than those encountered in identification of oncogenes where excess function is a characteristic of their involvement in human cancers. In addition, function of a number of different chromosome 5 genes may well be disturbed by deletions in colorectal cancers (as suggested in Chapter 5) since reduction in gene expression will result for those genes which have been rendered hemizygous by deletion without reduplication. Hence proving that a candidate gene is indeed *APC* may well be problematical and it could be some years before cloning and full characterisation of the gene is achieved.

9.6 Aspects of future research in the molecular genetics of colorectal cancer.

For patients affected by specific cancers such as Hodgkins' lymphoma, teratoma and some childhood leukaemias, there have been great advances made in recent years in the results of treatment, with potential cure a realistic goal. However, there has been little, if any, overall progress made against cancer in the last 20 years (Bailar 1986). In the United States over the 20 years from 1962-1982, there has been a 56% increase in the number of deaths due to cancer (278,562-433,795). The crude mortality from cancer increased by 25% and age-adjusted cancer mortality by 8.7% while the age-adjusted incidence of cancer over the same time period rose by 8.5%. This parallel rise of cancer incidence and death rates occurred in the face of a massive increase in expenditure on cancer research in the United States. Although there are clearly some successes, Bailar believes that we are losing the war against cancer (Bailar 1986). As discussed in Chapter 1 of this thesis, little impact has been made on death rates from colorectal cancer in the

United Kingdom and although there has been a modest reduction in the incidence of colorectal cancer incidence over the last few years in the United States (Bailar 1986), the world-wide incidence of the disease is increasing and it appears that we are losing ground against colorectal cancer in particular.

Research efforts to date have largely been directed at developing and optimising empiric treatment protocols derived from clinical or laboratory observations rather than any specific therapy based on knowledge of the biology of cancer. Understanding the fundamental molecular mechanisms involved in the initiation, proliferation and metastasising properties of cancer will hopefully lead to a specific and a more structured approach to cancer and to the development of anti-cancer treatments. Examples of such an approach are already in evidence. The recent discovery of the involvement of p53 gene mutations in colorectal and lung cancer (Baker 1989, Iggo 1990) could result in clinical benefit since highly specific monoclonal antibodies could be developed to recognise the mutant p53 gene product, allowing imaging of tumour tissue and even targeted delivery of chemotherapeutic agents. It has also been proposed that it may be possible to mobilise the human immune system against cancer cells by immunization with a specific mutant p53 antigen (Bodmer personal communication 1990).

A further exciting prospect is that if the bulk of colorectal cancer incidence is indeed concentrated in a minority of the population due to genetic predisposition as suggested by Cannon-Albright (Cannon-Albright 1989), then molecular genetics may be able to identify specific gene defects and those individuals who carry them. This would allow targeted screening of a high risk population and intensive screening modalities could be employed which would not be possible for population screening. Large scale automated DNA analysis is already a practical proposition (Landegren 1988) and the entire population could be screened in the same manner as is already done with the neonatal Guthrie test. Carriers of specific gene defects known to predispose to colorectal cancer could be identified and 'flagged' for colonoscopic screening on entering pre-defined high risk age groups. An alternative would be to screen relatives of patients affected by colorectal cancer for gene defects and then offer clinical screening to those with the cancer predisposition gene shown by DNA analysis.

The study of the biology of colorectal cancer and of the inheritance of colorectal cancer susceptibility using modern techniques of molecular genetics promises much for the future. This project has demonstrated that colorectal cancer risk for individuals at risk of FAP can be qualitatively assessed and appropriate clinical decisions made based on that risk. It is likely that the development of large bowel cancer in individuals affected by FAP can be completely avoided. It can only

be hoped that reduction in mortality from non-FAP colorectal cancer can also be achieved.

CHAPTER 10

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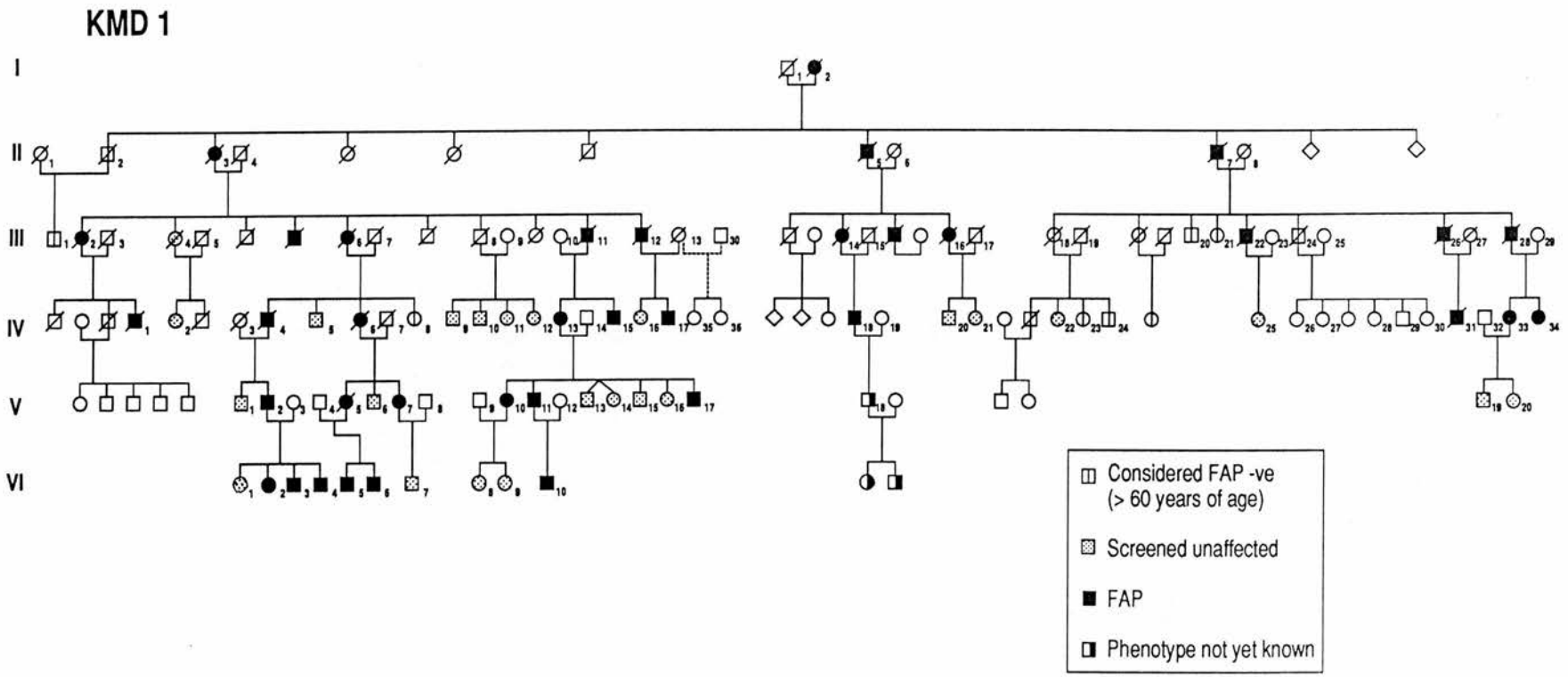
APPENDIX A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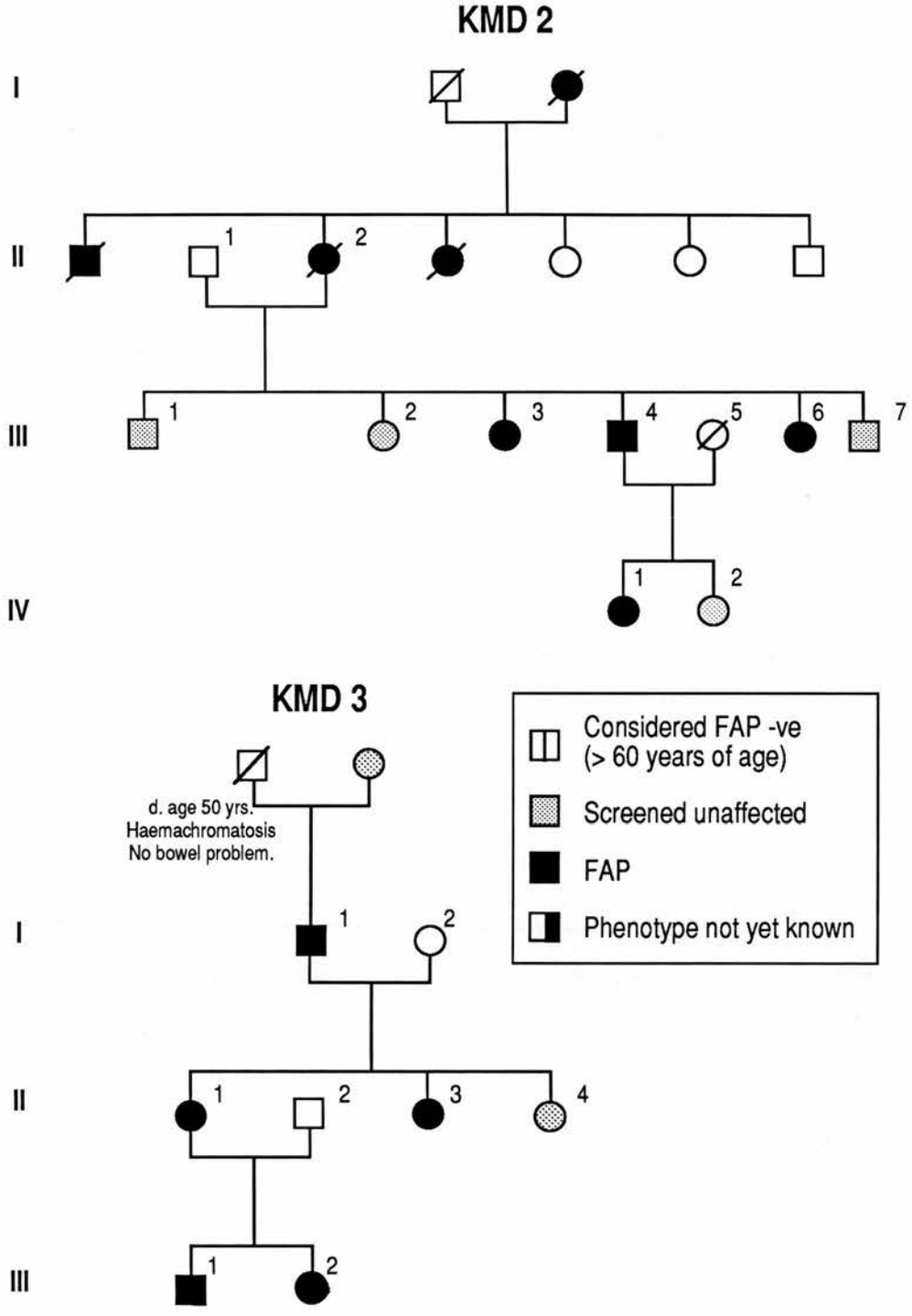
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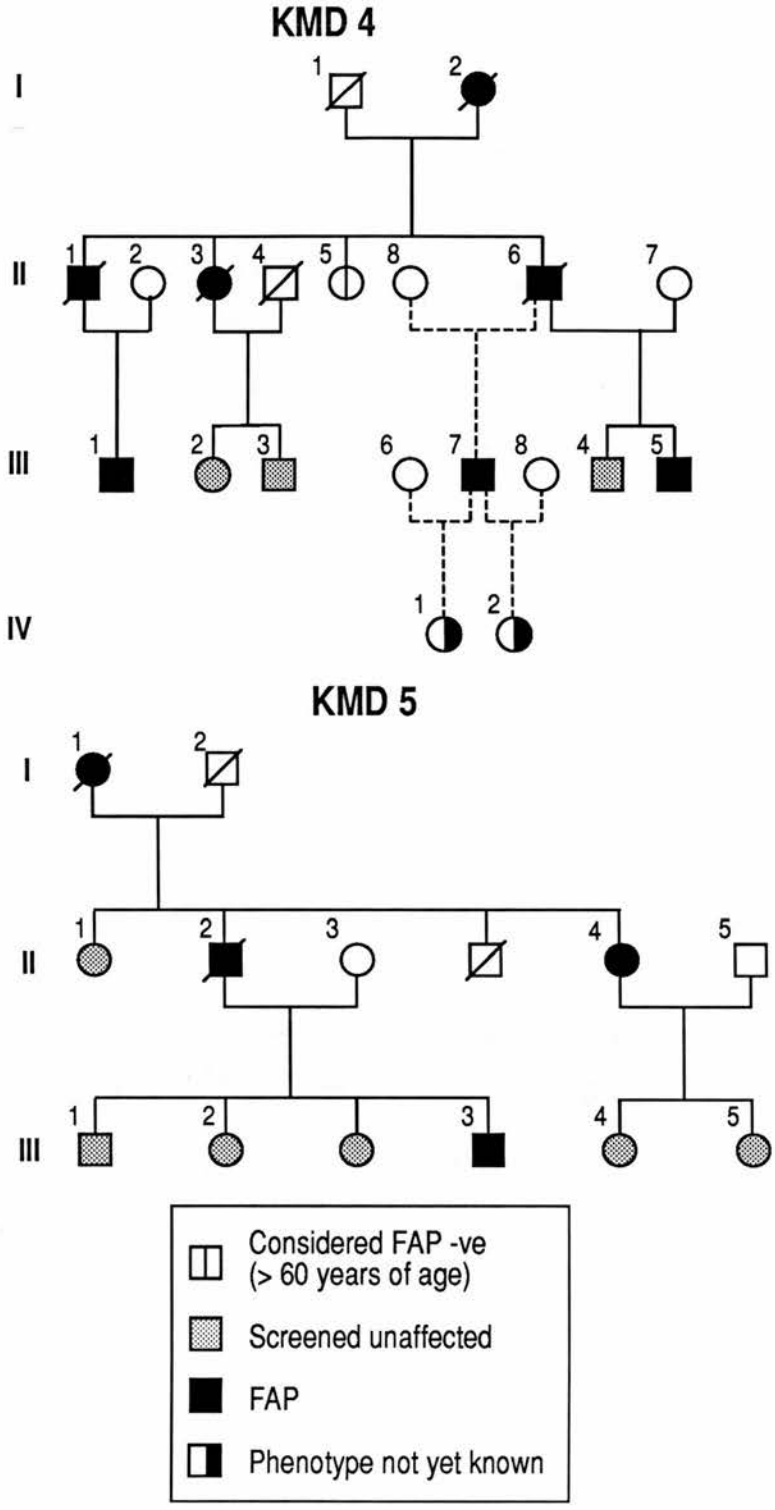
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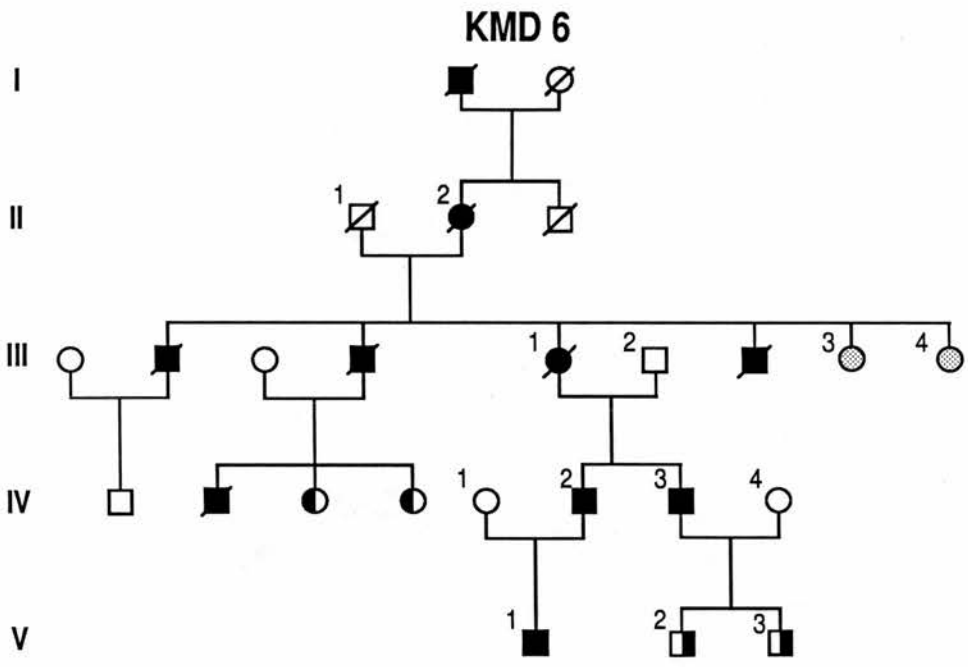
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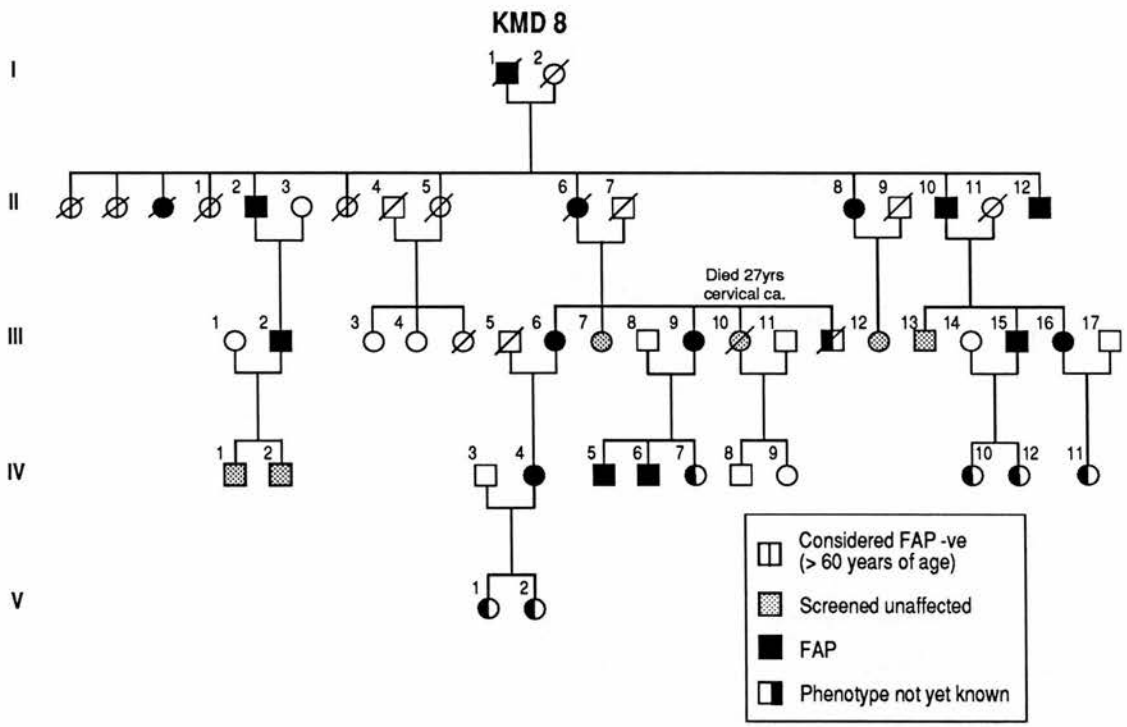
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FAP
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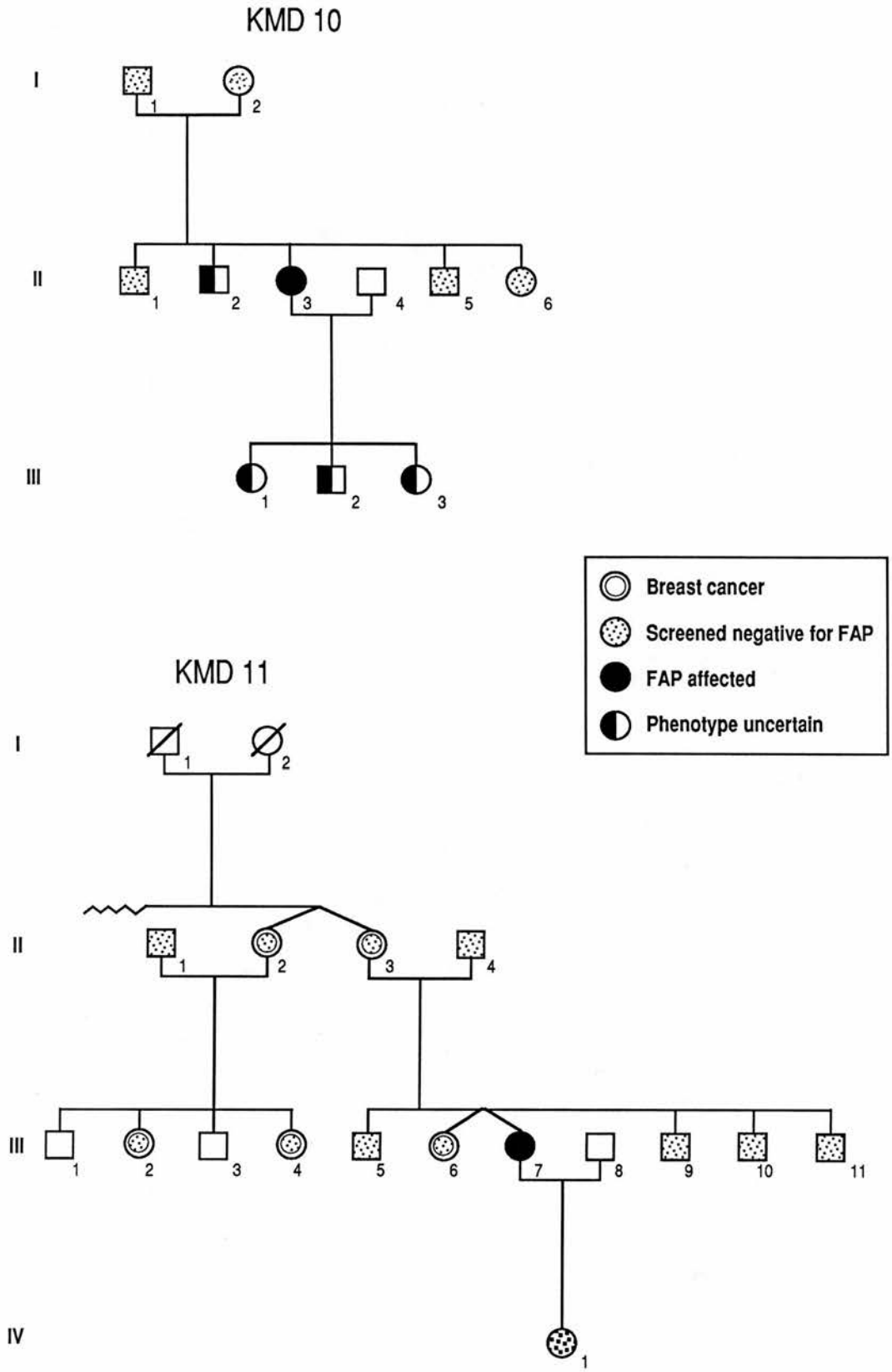
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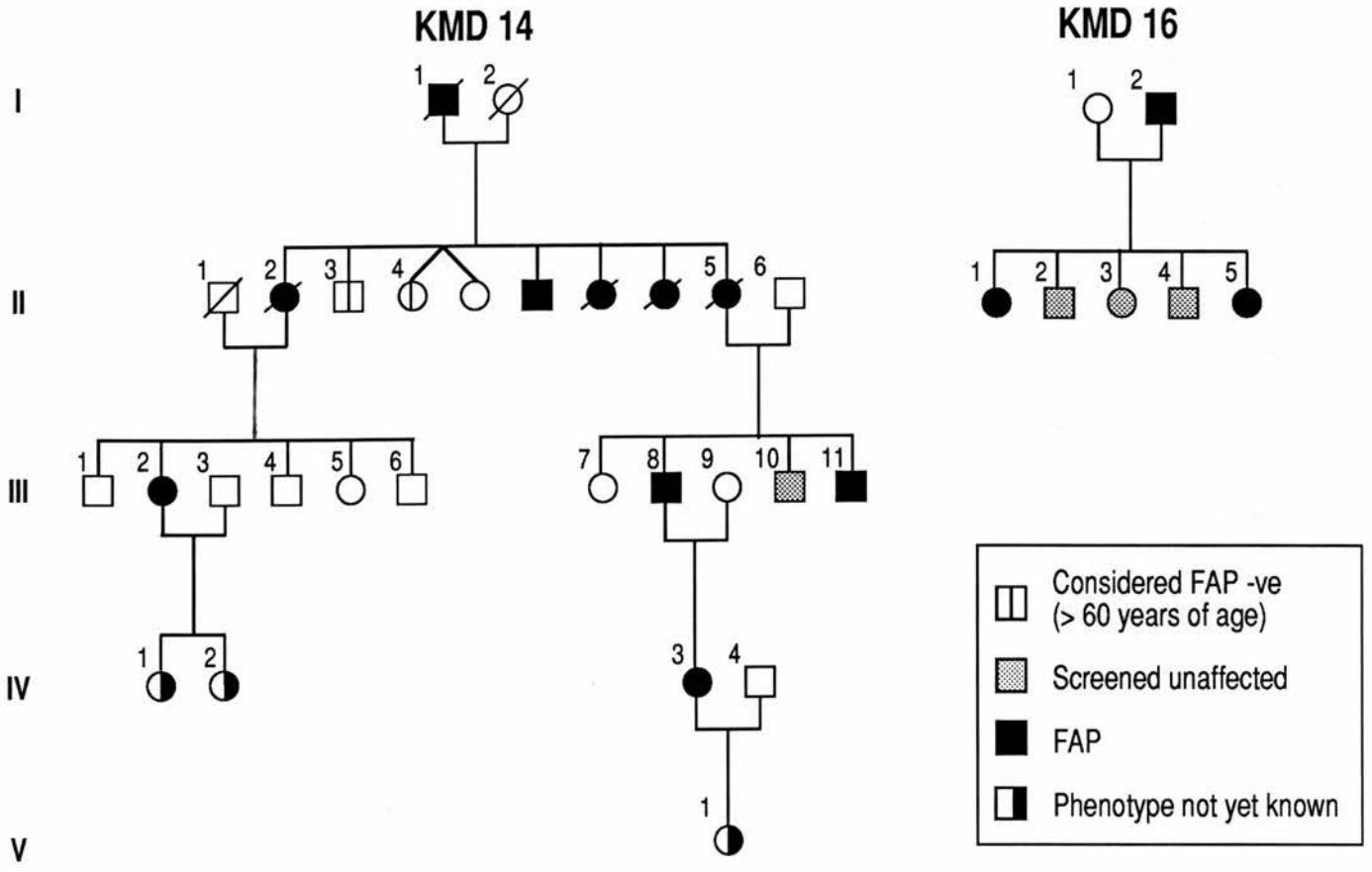
6
7
8
9
10
11
IV
1
Breast cancer
Screened negative for FAP
FAP affected
Phenotype uncertain


KMD 14
KMD 16
I
II
III
IV
V
Considered FAP -ve
(> 60 years of age)
Screened unaffected
FAP
Phenotype not yet known

APPENDIX B

KMD1 FILE

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	YN48	L562	EF54	ECB
1-1		0.00	M			-							
1-2		0.00	F			+							
2-1		0.00	F			-							
2-2		0.00	M	1-1	1-2	-							
2-3		0.00	F	1-1	1-2	+							
2-4		0.00	M			-							
2-5		0.00	M	1-1	1-2	+							
2-6		0.00	F			-							
2-7		0.00	M	1-1	1-2	+							
2-8		0.00	F			-							
3-1		0.00	M	2-2	2-1	-	2/2	2/2		1/2	1/1	1/2	1/2
3-2		0.00	F	2-4	2-3	+							
3-3		0.00	M			-							
3-4		0.00	F	2-4	2-3	-							
3-5		0.00	M			-							
3-6		0.00	F	2-4	2-3	+							
3-7		0.00	M			-							
3-8		0.00	M	2-4	2-3	-							
3-9		0.00	F			-	2/2	1/2		1/2	1/1	1/2	1/2
3-10		0.00	F			-	1/2	1/1		1/1	1/1	2/2	1/2
3-11		0.00	M	2-4	2-3	+		2/2					
3-12		0.00	M	2-4	2-3	+							
3-13		0.00	F			-							
3-14		0.00	F	2-5	2-6	+							
3-15		0.00	M			-							
3-16		0.00	F	2-5	2-6	+							
3-17		0.00	M			-							
3-18		0.00	F	2-7	2-8	-							
3-19		0.00	M			-							
3-20		0.00	M	2-7	2-8	-	2/2	1/2		1/2	1/1	2/2	2/2
3-21		0.00	F	2-7	2-8	-	2/2	1/2		2/2	1/1	2/2	2/2
3-22		0.00	M	2-7	2-8	+							
3-23		0.00	F			-	2/2	2/2		1/2	1/1	2/2	1/2
3-24		0.00	M	2-7	2-8	-							
3-25		0.00	F			-	2/2	2/2		1/1	1/2	1/1	1/1
3-26		0.00	M	2-7	2-8	+							
3-27		0.00	F			-							
3-28		0.00	M	2-7	2-8	+		2/2					
3-29		0.00	F			-	2/2	1/2		1/2	1/2	2/2	2/2
3-30		0.00	M			-							
4-1		0.00	M	3-3	3-2	+		2/2					
4-2		0.00	F	3-5	3-4	-	2/2	1/2		1/2	1/1	1/2	2/2
4-3		0.00	F			-							
4-4		0.00	M	3-7	3-6	+							
4-5		0.00	M	3-7	3-6	-	1/2	2/2		2/2	1/1	2/2	2/2
4-6		0.00	F	3-7	3-6	+							
4-7		0.00	M			-							
4-8		0.00	F	3-7	3-6	-	2/2	1/2		1/2	1/1	2/2	2/2
4-9		0.00	M	3-8	3-9	-	2/2	2/2		1/2	1/1	2/2	1/1
4-10		0.00	M	3-8	3-9	-	2/2	1/2		1/1	1/1	2/2	1/2
4-11		0.00	F	3-8	3-9	-	2/2	1/2		1/1	1/1	2/2	1/2
4-12		0.00	F	3-8	3-9	-	2/2	2/2		1/1	1/1	1/2	2/2
4-13		0.00	F	3-11	3-10	+	1/1	1/2		1/2	1/2	2/2	2/2
4-14		0.00	M			-	2/2	2/2		1/1	1/2	2/2	1/2
4-15		0.00	M	3-11	3-10	+	1/1	1/2		1/2	1/2	2/2	2/2
4-16		0.00	F	3-12	3-13	-	2/2	2/2		1/2	1/1	2/2	1/2

For all pedigrees, probe codes are as follows:

C11=C11P11; P27A=pi227/Pst1; P27B=pi227/Bcl1;

YN48=YN5.48; EF54=EF5.44; ECB=ECB27.

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	YN48	L562	EF54	ECB
4-20		0.00	M	3-17	3-16	-	2/2	2/2		1/1	1/1	1/2	
4-21		0.00	F	3-17	3-16	-	2/2	2/2		1/1	1/1	1/2	
4-22		0.00	F	3-19	3-18	-	1/2	2/2		1/2	1/1	2/2	2/2
4-23		0.00	F	3-19	3-18	-	2/2	1/2				2/2	2/2
4-24		0.00	M	3-19	3-18	-	1/2	2/2		1/2	1/1	2/2	
4-25		0.00	F	3-22	3-23	-	2/2	1/2		1/1	1/1	2/2	1/2
4-26		0.00	F	3-24	3-25	-	2/2	2/2		1/2	1/2	1/2	1/2
4-27		0.00	F	3-24	3-25	-	2/2	2/2		1/2	1/1	1/2	1/1
4-28		0.00	F	3-24	3-25	-	2/2	2/2		1/1	1/1	1/1	1/2
4-29		0.00	M	3-24	3-25	-	2/2	2/2		1/1	1/1	1/1	1/2
4-30		0.00	F	3-24	3-25	-	2/2	2/2		1/2	1/2	1/2	1/1
4-31		0.00	M	3-26	3-27	+		2/2					
4-32		0.00	M			-	2/2	2/2		2/2	1/1	2/2	1/1
4-33		0.00	F	3-28	3-29	+	1/2	1/2		2/2	2/2	2/2	2/2
4-34		0.00	F	3-28	3-29	+	1/2	2/2		2/2	2/2	2/2	2/2
4-35		0.00	F	3-30	3-13	-	2/2	2/2		2/2	1/1	2/2	1/2
4-36		0.00	F	3-30	3-13	-	2/2	2/2		1/2	1/1	2/2	1/1
5-1		0.00	M	4-4	4-3	-	2/2	2/2		1/2	1/1	1/2	2/2
5-2		0.00	M	4-4	4-3	+	2/2	1/2		2/2	1/2	1/2	2/2
5-3		0.00	F			-	2/2	1/1		2/2	1/1	1/2	1/2
5-4		0.00	M			-	2/2	2/2		1/2	1/1	2/2	1/2
5-5		0.00	F	4-7	4-6	+							
5-6		0.00	M	4-7	4-6	-	2/2	2/2		1/2	1/2	1/2	1/1
5-7		0.00	F	4-7	4-6	+	1/2	2/2		2/2	1/2	1/2	1/2
5-8		0.00	M			-	1/2	2/2		1/2	1/1	2/2	1/2
5-9		0.00	M			-	2/2	1/1		1/2	1/1	2/2	1/2
5-10		0.00	F	4-14	4-13	+	1/2	2/2		1/2	1/2	2/2	2/2
5-11		0.00	M	4-14	4-13	+	1/2	2/2		1/2	1/2	2/2	2/2
5-12		0.00	F			-	2/2	1/2		1/2	1/1	2/2	1/2
5-13		0.00	M	4-14	4-13	-	1/2	1/2		1/1	1/2	2/2	1/2
5-14		0.00	F	4-14	4-13	-	1/2	1/2		1/1	1/2	2/2	1/2
5-15		0.00	M	4-14	4-13	-	1/2	1/2		1/1	1/1	2/2	2/2
5-16		0.00	F	4-14	4-13	-	1/2	1/2		1/1	1/2	2/2	1/2
5-17		0.00	M	4-14	4-13	+	1/2	2/2		1/2	1/2	2/2	2/2
5-18		0.00	M	4-18	4-19		2/2	2/2		1/1	1/2	2/2	2/2
5-19		0.00	M	4-32	4-33	-	2/2	1/2		2/2	1/2	2/2	1/2
5-20		0.00	F	4-32	4-33	-	2/2	1/2		2/2	1/2	2/2	1/2
6-1		0.00	F	5-2	5-3	+	2/2	1/1		2/2	1/1	1/2	1/2
6-2		0.00	F	5-2	5-3	+	2/2	1/1		2/2	1/2	2/2	1/2
6-3		0.00	M	5-2	5-3	+	2/2	1/1		2/2	1/2	1/2	2/2
6-4		0.00	M	5-2	5-3	+	2/2	1/1		2/2	1/2	2/2	1/2
6-5		0.00	M	5-4	5-5	+	1/2	2/2		2/2	1/2	2/2	2/2
6-6		0.00	M	5-4	5-5	+	1/2	2/2		2/2	1/2	2/2	2/2
6-7		0.00	M	5-8	5-7	-	2/2	2/2		1/2	1/1	1/2	1/2
6-8		0.00	F	5-9	5-10	-	2/2	1/2		1/2	1/1	2/2	1/2
6-9		0.00	F	5-9	5-10	-	2/2	1/2		1/2	1/1	2/2	2/2
6-10		0.00	M	5-11	5-12	+	1/2	2/2		1/2	1/2	2/2	1/2

KMD2 FILE

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	YN48	L562	EF54	ECB
2-1		0.00	M			-	1/1		1/3	2/2	1/1	2/2	1/2
2-2		0.00	F			+							
3-1		0.00	M	1-1	1-2	-	1/1		3/3	2/2	1/1	1/2	1/2
3-2		0.00	F	1-1	1-2	-	1/1		1/3	2/2	1/1	1/2	2/2
3-3		0.00	F	1-1	1-2	+	1/2		3/3	1/2	1/1	2/2	1/2
3-4		0.00	M	1-1	1-2	+	1/2		1/3	1/2	1/1	2/2	2/2
3-5		0.00	F			-	1/2		3/3	1/2	1/1	2/2	
3-6		0.00	F	1-1	1-2	+	1/1		1/3	1/2	1/1	2/2	2/2
3-7		0.00	M	1-1	1-2	-	1/1		1/3	2/2	1/1	1/2	1/2
4-1		0.00	F	2-4	2-5	+	2/2		3/3	1/2	1/1	2/2	2/2
4-2		0.00	F	2-4	2-5	-	1/2		1/3	2/2	1/1	2/2	2/2

KMD3 FILE

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	YN48	L562	EF54	ECB
1-1		0.00	M			+	2/2		2/2	1/2	1/1	1/2	1/2
1-2		0.00	F			-	2/2		1/3	1/2	1/1	2/2	1/2
2-1		0.00	F	1-1	1-2	+	2/2		2/3	1/2	1/1	1/2	1/2
2-2		0.00	M			-	2/2		2/3	1/1	1/1	1/2	1/1
2-3		0.00	F	1-1	1-2	+	2/2		1/2	1/2	1/1	1/2	2/2
2-4		0.00	F	1-1	1-2	-	2/2		1/2	1/1	1/1	2/2	1/2
3-1		0.00	M	2-2	2-1	+	2/2		2/2	1/2	1/1	1/2	1/2
3-2		0.00	F	2-2	2-1	+	2/2		2/3	1/2	1/1	1/1	1/2

KMD4 FILE

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	YN48	L562	EF54	ECB
1-1		0.00	M			-							
1-2		0.00	F			+							
2-1		0.00	M	1-1	1-2	+							
2-2		0.00	F			-	2/2	2/2	1/3	1/2	1/1	1/2	2/2
2-3		0.00	F	1-1	1-2	+							
2-4		0.00	M			-							
2-5		0.00	F	1-1	1-2	-	2/2	1/2	2/3	2/2	1/1	2/2	1/2
2-6		0.00	M	1-1	1-2	+							
2-7		0.00	F			-	2/2	1/2	2/3	1/2	1/1	2/2	1/2
2-8		0.00	F			-	2/2	1/2	2/3	1/2	1/1	2/2	2/2
3-1		0.00	M	2-1	2-2	+	2/2	2/2	3/3	2/2	1/1	1/2	1/2
3-2		0.00	F	2-4	2-3	-	2/2	2/2	1/3	1/1	1/1	1/2	1/1
3-3		0.00	M	2-4	2-3	-	2/2	2/2	1/3	1/2	1/1	2/2	1/1
3-4		0.00	M	2-6	2-7	-	2/2	1/2	2/3	1/2	1/1	2/2	1/2
3-5		0.00	M	2-6	2-7	+	2/2	2/2	3/3	2/2	1/1	2/2	1/1
3-6		0.00	M	2-6	2-8	+	2/2	1/2	2/3	2/2	1/1	2/2	1/2
3-7		0.00	F			-		2/2	2/3				
3-8		0.00	F			-							
4-1		0.00	F	3-6	3-7			2/2	2/3				
4-2		0.00	F	3-6	3-8		2/2	1/2	2/3	1/2		2/2	1/2

KMD5 FILE

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	TN48	L562	EF54	ECB
1-1		0.00	F			+							
1-2		0.00	M			-							
2-1		0.00	F	1-2	1-1	-	2/2		2/2	2/2	1/1	2/2	2/2
2-2		0.00	M	1-2	1-1	+							
2-3		0.00	F			-	2/2		1/2	1/1	1/1	2/2	2/2
2-4		0.00	F	1-2	1-1	+	2/2		2/2	2/2	1/1	2/2	2/2
2-5		0.00	M			-	2/2		1/3	1/2	1/1	2/2	1/2
3-1		0.00	M	2-2	2-3	-	2/2		1/2	1/2	1/1	2/2	2/2
3-2		0.00	F	2-2	2-3	-	2/2		1/2	1/2	1/1	2/2	2/2
3-3		0.00	M	2-2	2-3	+	2/2		1/2	1/2	1/1	2/2	2/2
3-4		0.00	F	2-5	2-4	-	2/2		1/2	1/2	1/1	2/2	2/2
3-5		0.00	F	2-5	2-4	-	2/2		1/2	1/2	1/1	2/2	2/2

KMD6 FILE

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	TN48	L562	EF54	ECB
1-1		0.00	M			-							
1-2		0.00	F			+							
2-1		0.00	F	1-1	1-2	+			2/3				
2-2		0.00	M			-	2/2		1/2	2/2	1/1	2/2	1/2
2-3		0.00	F	1-1	1-2	-	2/2		2/2	1/2	1/1	2/2	2/2
2-4		0.00	F	1-1	1-2	-	2/2		2/2	1/2	1/1	1/2	1/2
3-1		0.00	F			-	1/1		2/2	1/2	1/1	1/2	1/2
3-2		0.00	M	2-2	2-1	+	2/2		1/3	1/2	1/1	2/2	1/2
3-3		0.00	M	2-2	2-1	+	2/2		2/3	1/2	1/1	2/2	2/2
3-4		0.00	F			-	1/2		3/3	2/2	1/2	2/2	2/2
4-1		0.00	M	3-2	3-1	+	1/2		1/2	1/2	1/1	1/2	1/1
4-2		0.00	M	3-3	3-4		1/2		2/3	1/2	1/2	2/2	2/2
4-3		0.00	M	3-3	3-4		2/2		2/3	2/2	1/1	2/2	2/2

KMD8 FILE

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	YN48	L562	EF54	ECB
1-1		0.00	M			+							
1-2		0.00	F			-							
2-1		0.00	F	1-1	1-2	-	2/2		3/3	2/2	1/1	2/2	1/2
2-2		0.00	M	1-1	1-2	+							
2-3		0.00	F			-	1/2		1/3	1/2	1/1	2/2	2/2
2-4		0.00	M			-							
2-5		0.00	F	1-1	1-2	-							
2-6		0.00	F	1-1	1-2	+							
2-7		0.00	M			-							
2-8		0.00	F	1-1	1-2	+	2/2		3/3	1/2	1/2	2/2	2/2
2-9		0.00	M			-							
2-10		0.00	M	1-1	1-2	+	2/2		2/3	2/2	1/2	2/2	1/2
2-11		0.00	F			-							
2-12		0.00	M	1-1	1-2	+	2/2		2/3	1/2	1/2	2/2	2/2
3-1		0.00	F			-	2/2		1/2	1/1	1/1	2/2	1/2
3-2		0.00	M	2-2	2-3	+	1/2		1/3	1/2	1/2	2/2	2/2
3-3		0.00	F	2-4	2-5	-	2/2		2/3	1/2	1/1	2/2	1/2
3-4		0.00	F	2-4	2-5	-	2/2		2/3	2/2	1/1	2/2	1/2
3-5		0.00	M			-							
3-6		0.00	F	2-7	2-6	+	2/2		1/3	2/2	1/2	2/2	2/2
3-7		0.00	F	2-7	2-6	-	2/2		1/2	2/2	1/1	2/2	1/2
3-8		0.00	M			-	2/2		2/3	1/1	1/1	2/2	2/2
3-9		0.00	F	2-7	2-6	+	2/2		1/3	2/2	1/2	1/2	2/2
3-10		0.00	F	2-7	2-6	-							
3-11		0.00	M			-	1/2		3/3	2/2	1/1	2/2	1/2
3-12		0.00	F	2-9	2-8	-	2/2		2/3	1/2	1/1	2/2	1/2
3-13		0.00	M	2-10	2-11	-	1/2		2/2	1/2	1/1	2/2	1/2
3-14		0.00	F			-	2/2		1/3	2/2	1/1	1/1	1/2
3-15		0.00	M	2-10	2-11	+	1/2		2/3	1/2	1/2	2/2	2/2
3-16		0.00	F	2-10	2-11	+	1/2		2/3	1/2	1/2	2/2	2/2
3-17		0.00	M			-	2/2		1/3	2/2	1/1	1/2	1/2
4-1		0.00	M	3-2	3-1	-	1/2		1/2	1/1	1/1	2/2	2/2
4-2		0.00	M	3-2	3-1	-	1/2		1/2	1/1	1/1	2/2	2/2
4-3		0.00	M			-							
4-4		0.00	F	3-5	3-6	+	2/2		1/3	2/2	1/2	1/2	2/2
4-5		0.00	M	3-8	3-9	+	2/2		1/2	1/2	1/2	2/2	2/2
4-6		0.00	M	3-8	3-9	+	2/2		3/3	1/2	1/2	2/2	2/2
4-7		0.00	F	3-8	3-9		2/2		2/3	1/2	1/2	2/2	2/2
4-8		0.00	M	3-11	3-10	-	2/2		2/3	2/2	1/1	2/2	1/2
4-9		0.00	F	3-11	3-10	-	1/2		1/3	2/2	1/1	2/2	1/2
4-10		0.00	F	3-15	3-14		2/2		1/3	2/2	1/2	1/2	1/2
4-11		0.00	F	3-17	3-16		2/2		1/3	1/2	1/2	2/2	2/2
5-1		0.00	F	4-3	4-4								
5-2		0.00	F	4-3	4-4								

KMD14 FILE

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	YN48	L562	EF54	ECB
1-1		0.00	M			+							
1-2		0.00	F			-							
2-1		0.00	M			-							
2-2		0.00	F	1-1	1-2	+							
2-3		0.00	M	1-1	1-2	-							
2-4		0.00	F	1-1	1-2	-							
2-5		0.00	F	1-1	1-2	+							
2-6		0.00	M			-	1/2		2/3	1/2	1/1	1/1	1/2
3-1		0.00	M	2-1	2-2	-							
3-2		0.00	F	2-1	2-2	+							
3-3		0.00	M			-							
3-4		0.00	M	2-1	2-2								
3-5		0.00	F	2-1	2-2								
3-6		0.00	M	2-1	2-2								
3-7		0.00	F	2-6	2-5	-	1/1		3/3	1/2	1/2	1/1	2/2
3-8		0.00	M	2-6	2-5	+	1/2		3/3	1/1	1/1	1/1	2/2
3-9		0.00	F			-							
3-10		0.00	M	2-6	2-5	-	1/1		3/3	1/2	1/2	1/1	2/2
3-11		0.00	M	2-6	2-5								
4-1		0.00	F	3-3	3-2								
4-2		0.00	F	3-3	3-2								
4-3		0.00	F	3-8	3-9								

KMD16 FILE

ID	D NAME	AGE	SEX	FATH	MOTH	FAP	C11	P27A	P27B	YN48	L562	EF54	ECB
1-1		0.00	F			-			2/3	1/2	1/1	1/2	1/2
1-2		0.00	M			+	2/2		2/3	1/2	1/1	2/2	1/1
2-1		0.00	F	1-2	1-1	+	1/2		2/3	2/2	1/1	2/2	1/2
2-2		0.00	M	1-2	1-1	-	2/2		2/3	1/1	1/1	1/2	1/1
2-3		0.00	F	1-2	1-1	-	1/2		2/2	1/1	1/1	1/2	1/2
2-4		0.00	M	1-2	1-1	-	1/2		2/2	1/2	1/1	2/2	1/2
2-5		0.00	F	1-2	1-1	+	1/2		2/3	1/2	1/1	2/2	1/2

GENOMICS 5, 350–353 (1989)

Linkage Analysis in Familial Adenomatous Polyposis: Order of C11P11 (*D5S71*) and π227 (*D5S37*) Loci at the *apc* Gene

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Received February 15, 1989

Linkage analysis with DNA probes C11P11 and π227 is reported in six Scottish families with familial adenomatous polyposis. Two families were informative for C11P11 and all six were at least partly informative for π227. Two C11P11–*apc* and two π227–*apc* recombinants were identified and one of these was recombinant for both C11P11–*apc* and π227–*apc*. A further possible combined C11P11–*apc* and π227–*apc* recombination event was also identified. Peak lod score for linkage of C11P11 to *apc* was 5.80 at a recombination fraction (θ) of 0.069 (95% probability limits 0.012–0.191) and for linkage of π227 to *apc* was 3.19 at θ = 0.110 (95% probability limits 0.023–0.286). Peak lod score for linkage of C11P11 to π227 was 1.79 at θ = 0.00. The data support a gene order of π227–C11P11–*apc*.

INTRODUCTION

Familial adenomatous polyposis (APC) is an uncommon autosomal dominant condition characterized by the development of large numbers of intestinal adenomas with a very high risk of progression to colorectal carcinoma if untreated (Muto *et al.*, 1975). Gardner syndrome (Gardner, 1951) consists of multiple osteomas, epidermoid cysts, and desmoid tumors in addition to familial polyposis, but clinically appears to be the same condition (Bulow, 1987). Congenital hypertrophy of the retinal pigment epithelium (CHRPE) is also part of the APC syndrome (Blair and Trempe, 1980; Traboulsi *et al.*, 1987) and is an important phenotypic marker of the disease in children because screening of the colon is not usually performed before puberty.

The recent establishment of genetic linkage of APC to chromosomal region 5q21–22 (Bodmer *et al.*, 1987; Leppert *et al.*, 1987) using the DNA probe C11P11 (*D5S71*) is of great clinical and scientific importance. Presymptomatic prediction of the disease is now possible, particularly following a report of linkage to APC with another probe, π227 (*D5S37*) (Meera Khan *et al.*, 1988), which is more polymorphic than C11P11. C11P11 and π227 have been localized by physical methods to chromosome 5q21–22 (Bodmer *et al.*, 1987; Alitalo *et al.*, 1987). Gene loss on chromosome 5q has been demonstrated in sporadic colorectal cancer (Solomon *et al.*, 1987) and thus identification of the *apc* gene may help advance our understanding of the role of putative tumor suppressor genes (Knudson, 1985) in colorectal carcinogenesis. Defining gene order and genetic distances in the region of chromosome 5 which includes the *apc* gene, C11P11, and π227 has great clinical significance and is a first step toward the goal of isolating the *apc* gene itself. However, C11P11 is relatively uninformative and only one C11P11–*apc* recombinant has been reported (Aldred *et al.*, 1988). No π227 recombination data were provided in that report.

We now report linkage analysis in six Scottish families with APC by using C11P11 and π227 and have identified important C11P11–*apc* recombinants and π227–*apc* recombinants. Estimates of genetic distances and a preferred order of the *apc*, C11P11, and π227 loci are given.

METHODS

Family data were drawn from the MRC Human Genetics Unit Registry and were fully documented for clinical and pathological status. Six apparently unrelated families, comprising 112 typed individuals, were studied. Penetrance was assigned as 1.0 for ages over 25 years, as the disease was apparent on examination of the colon in all affected individuals in these families before this age. At-risk individuals who were under 25 years and negative for colonic adenomas were considered negative for APC only if examination of the optic fundus by an experienced ophthalmologist demonstrated no CHRPE lesions. The phenotypes of 8 affected and 5 unaffected children under 15 years were

0888-7543/89 $3.00

ascertained by fundoscopy alone, as screening of the colon was not carried out in patients under this age. Four or more CHRPE lesions in one eye or three lesions involving both eyes were considered positive and complete absence of CHRPE lesions was considered negative for APC. Only children who had an affected parent positive for the CHRPE phenotype were screened in this way, as we have found the presence or absence of CHRPE lesions in affected individuals within any one family to cosegregate with the colonic manifestations of the disease (unpublished data).

DNA was purified from whole blood by lysis in 2% SDS, 0.1 *M* Tris–HCl, pH 7.5, 20 m*M* NaCl, 1 m*M* EDTA for 5 min and then phenol/chloroform extracted and NH_4COOH/ethanol precipitated. Spooled DNA was resuspended in 10 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 10 m*M* EDTA and treated with 50 $\mu g\ ml^{-1}$ RNase followed by 100 $\mu g\ ml^{-1}$ proteinase K at 37°C overnight in the presence of 0.2% SDS. Two further phenol/chloroform extractions and then NH_4COOH/ethanol precipitation were carried out, and spooled DNA was dissolved in 10 m*M* Tris–HCl, pH 7.9, 0.5 m*M* EDTA (TE). Epstein–Barr virus-transformed lymphoblastoid cell lines were also set up from all family members. DNA was extracted from archival formalin-fixed paraffin-embedded normal material from a number of deceased important family members by using the following method, slightly modified from those previously described (Dubeau *et al.*, 1986; Warford *et al.*, 1988). Histologically confirmed normal tissue was sectioned at 5 μm on the microtome to give around 100 mg of tissue including residual paraffin. Paraffin was dissolved in 10 ml xylene with three xylene washes. Xylene was removed in 100% ethanol and the tissue progressively rehydrated in 99% ethanol (3 washes), 95% ethanol (2 washes), 70% ethanol (1 wash), and distilled water (1 wash). The tissue was then resuspended, proteinase K treated, phenol/chloroform extracted, and ethanol precipitated as for whole blood. DNA was recovered by centrifugation at 12K for 30 min, resuspended in TE, treated with RNase and proteinase K, and reprecipitated with ethanol as before. The pellet was then dissolved in TE.

Five micrograms of purified DNA was digested with the appropriate restriction endonuclease (*Taq*1 for C11P11; *Pst*1, *Bcl*1, *Mbo*1, or *Bst*X1 for π227), fractionated in agarose gel, and transferred to a nylon filter according to manufacturers' instructions. Filters were prehybridized in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 10% dextran sulfate for 2 h. Probe DNA was radiolabeled to a specific activity of 5×10^8–1×10^9 cpm μg^{-1} by oligonucleotide labeling (Feinberg and Vogelstein, 1983) and hybridized to the filter overnight at 65°C in the presence of salmon sperm DNA. Two 5-min stringency washes of 2× SSC and 0.1% SDS followed by 2× 5 min 0.1× SSC and 0.1% SDS were carried out at 65°C prior to autoradiography at −70°C for 24–48 h.

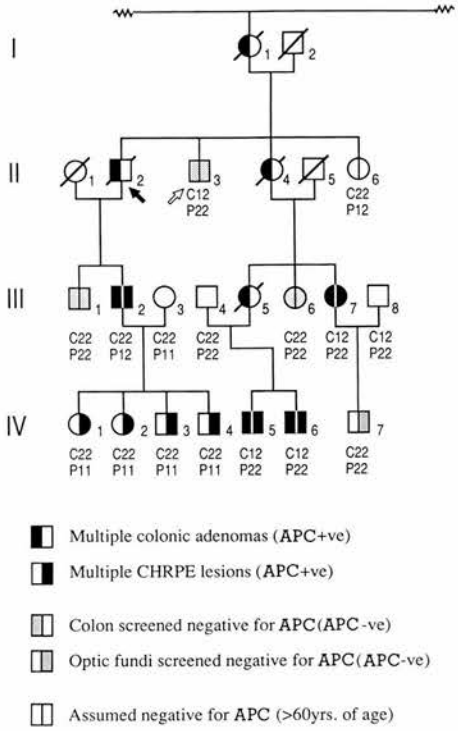

FIG. 1. Part of a large family (KMD1) showing *Taq*1 C11P11 alleles (C1, 4.4 kb; C2, 3.9 kb) and *Pst*1 π227 alleles (P1, 3.0 kb; P2, 4.3 kb). C1 and P2 segregate with the *apc* gene in the other branches of this kindred. The black arrow indicates a likely site of recombination involving both C11P11–*apc* and π227–*apc*. The open arrow indicates another possible C11P11–*apc* and π227–*apc* recombination event. π227 polymorphisms using other restriction enzymes did not provide any additional information in KMD1. The use of the CHRPE phenotype has allowed the phase of the disease with regard to the π227 alleles in individuals IV 1, 2, 3, and 4 to be ascertained, thereby suggesting the most likely site of a combined C11P11–*apc*, π227–*apc* recombination event (black arrow).

Linkage analysis was carried out using the LINKAGE 4.7 group of programs (Lathrop *et al.*, 1985), and the 1-lod interval of support was taken as approximating 95% probability limits.

RESULTS

Restriction fragment length polymorphisms (RFLPs) for C11P11 and π227 have been described elsewhere (Bodmer *et al.*, 1987; Leppert *et al.*, 1987; Meera Khan *et al.*, 1988) and no new variants were observed in these pedigrees. The frequencies of RFLPs in a control sample of normal individuals from this area in Scotland were C11P11—*Taq*1 A1 0.14, A2 0.86 (100 chromosomes), and π227—*Pst*1 A1 0.25, A2 0.75 (66 chromosomes); *Bcl*1 A1 0.17, A2 0.46, A3 0.37 (54 chromosomes); *Bst*X1 A1 0.29, A2 0.71 (28 chromo-

somes); *Mbo* A1 0.25, A2 0.75 (20 chromosomes). C11P11 was informative in two families. In one large family (KMD1) the disease gene was linked to the less common (A1, 4.4 kb) allele, allowing a large amount of information to be gathered. π227 was at least partly informative in all six families with *Pst*1 and *Bcl*1 RFLPs. Two obligate C11P11–*apc* recombinants and two π227–*apc* obligate recombinants were identified. A section of a large family (KMD1) is shown in Fig. 1. One meiosis in which there is strong evidence of a single recombination event affecting both the C11P11 and the π227 loci relative to the *apc* gene is indicated. Another possible combined C11P11–*apc* and π227–*apc* recombinant is also indicated. These observations strongly suggest that C11P11 and π227 loci are on the same side of the *apc* gene.

Lod score calculations (Lathrop *et al.*, 1985) were carried out for linkage between the C11P11 (*D5S71*) and the *apc* loci, the π227 (*D5S37*) and the *apc* loci, and the C11P11 and the π227 loci. Maximum lod score (z) for C11P11–*apc* was 5.80 at a recombination fraction (θ) of 0.069 (95% probability limits 0.012–0.191) and for π227–*apc*, z was 3.19 at $\theta = 0.110$ (95% probability limits 0.023–0.286) (Table 1). No π227–C11P11 recombinants were identified due to the small number of meioses informative for both C11P11 and π227. Peak lod score for C11P11–π227 was 1.79 at $\theta = 0.00$. These data support a locus order of π227–C11P11–*apc*.

This study has made use of archival formalin-fixed, paraffin-embedded tissue specimens for genotyping of deceased individuals for linkage analysis. In each case where good-quality alleles were achieved, the expected genotypes were obtained. Tissue blocks ranged from 2 to 41 years of age. An average of 20 μg of DNA was recovered from 100-mg blocks of tissue, which included lymph node, thyroid, appendix, ovary, heart, and stomach. The different tissues gave comparable recoveries of DNA but the yield and quality of DNA were poorer for the older blocks. Autolysed tissue and tissues fixed in picric acid did not provide satisfactory yields of DNA. DNA from paraffin-embedded material was purified from 11 different blocks. Only 6 of these hybridized with π227 and gave good-quality allelic bands, but no satisfactory hybridization was obtained with C11P11. This appears to be a locus-specific problem, as C11P11 did not hybridize to the *Pst*1-digested DNA which had given bands for π227.

TABLE 1

Lod Scores for Linkage of C11P11 and π227 to *apc*

	Recombination fraction (θ)					
Pedigree	0.00	0.05	0.10	0.15	0.20	0.25
			C11P11			
KMD1	$-\infty$	4.14	4.25	4.00	3.57	3.02
KMD2	1.81	1.65	1.49	1.31	1.13	0.93
KMD3	0.00	0.00	0.00	0.00	0.00	0.00
KMD4	−0.02	−0.02	−0.02	−0.01	−0.01	−0.01
KMD5	−0.02	−0.02	−0.02	−0.01	−0.01	−0.01
KMD6	−0.01	−0.01	−0.01	0.00	0.00	0.00
Total	$-\infty$	5.74	5.69	5.29	4.68	3.93
			π227			
KMD1	$-\infty$	2.00	2.22	2.18	2.02	1.78
KMD2	0.18	0.17	0.16	0.15	0.13	0.12
KMD3	0.30	0.28	0.26	0.23	0.20	0.18
KMD4	0.29	0.24	0.19	0.15	0.11	0.07
KMD5	−0.22	−0.17	−0.13	−0.10	−0.07	−0.05
KMD6	$-\infty$	0.35	0.49	0.50	0.45	0.37
Total	$-\infty$	2.87	3.19	3.10	2.84	2.47

DISCUSSION

This study has identified important rare recombination events between the closely linked marker C11P11 and the *apc* loci. At least one of these events also involves the π227 locus and this strongly suggests that C11P11 and π227 do not flank the *apc* gene.

The frequency of *Taq*1 C11P11 alleles in this study is at variance with the findings of Bodmer *et al.* (1987). The 4.4-kb allele (A1) occurs with greater frequency in our control population. This concurs with the findings of Leppert *et al.* (1987) and Aldred *et al.* (1988). C11P11 may therefore be a more useful marker for clinical use than originally suggested. A substantial peak lod score of 5.80 has been achieved in this study for C11P11 at a recombination fraction of 7%. Although the confidence limits from these data are still wide, a combination of published C11P11 (Bodmer *et al.*, 1987; Leppert *et al.*, 1987; Aldred *et al.*, 1988) and π227 (Meera Khan *et al.*, 1988) linkage data with our own indicates that C11P11 and π227 are approximately 5 and 5–10 cM, respectively, from the *apc* gene. However, a C11P11–*apc* interval of 7 cM suggested by data in this study is supported by multilocus analysis data from Nakamura *et al.* (1988) that place C11P11 17 cM centromeric to a marker (YN548) that is 3 cM telomeric to the *apc* gene. Gene order therefore appears to be centromere–π227–C11P11–*apc*–telomere. The C11P11 locus is clearly much further from the *apc* gene than suggested by the original reports (Bodmer *et al.*, 1987; Leppert *et al.*, 1987), and this has important clinical implications for the use of this marker in disease state prediction of at-risk individuals. In addition, chromosome walking over such a distance would be impractical.

This study has made use of archival paraffin-embedded fixed tissue in linkage analysis. Clearly, the ability to genotype deceased members of a kindred from such material may open the door to linkage analyses in many conditions that have proved difficult in the past because of the early demise of affected individuals. However, some problems in the use of archival material as a source of DNA have been identified. We have confirmed previous findings that DNA cannot be recovered from tissue fixed in picric acid (Bouin's) or mercuric chloride (Zenker's) preservatives (Dubeau *et al.*, 1986). In addition, we have demonstrated a locus-specific problem with hybridization of the probe C11P11, despite apparently good-quality DNA as seen on ethidium bromide staining of the gel. This may prove a problem with other DNA probes. Despite these shortcomings, DNA recovery from archival material promises to be an invaluable tool in future linkage analyses and in analysis of archival tumor material where correlation of tumor progression with specific genetic events may be possible.

ACKNOWLEDGMENTS

Probe C11P11 was originally from P. Scambler, Department of Biochemistry, St. Mary's Hospital, London, and probe π227 from G. D. Stewart, HHMI, Ann Arbor, Michigan. We are indebted to Mr. A. A. Gunn and Dr. G. Sclare of Bangour General Hospital, West Lothian, and Professor C. I. Phillips, Department of Ophthalmology, Edinburgh University, for their invaluable help. Many thanks to R. de Mey and S. Collyer of the MRC Human Genetics Unit Registry and to E. Kay, who prepared the manuscript. This work is partly supported by the Cancer Research Campaign. M.G.D. is supported by a MRC Training Fellowship.

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Oncogene (1989), 4, 1169–1174

High frequency of *APC* loss in sporadic colorectal carcinoma due to breaks clustered in 5q21-22

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Familial adenomatous polyposis is transmitted by a gene (*APC*) located within 5q21-22. Hemizygous loss of at least a part of 5q has been reported in 19–36% of sporadic colorectal carcinomas. This suggests that an anti-oncogene is located on that chromosome arm, but the probes used previously gave little information on the status of *APC* in the tumours. Using DNA probes homologous to polymorphic sequences flanking and close to the *APC* locus we show that more than half of a large series of carcinomas had lost at least one flanking allele. Mapping of allele losses provides data that imply clustering of breakpoints in a 10–15 megabase region around *APC*. The commonest chromosome defect responsible for *APC* loss was interstitial deletion. Mitotic recombination or partial arm loss were less frequent mechanisms. Whole chromosome loss was rare. This pattern contrasts with that reported in acquired homozygosity at other anti-oncogene loci in sporadic tumours and implies that *APC* loss is an early event in colorectal carcinogenesis. This view is also supported by the observations that 5q21-22 loss occurs with similar frequency in DNA diploid and DNA aneuploid tumours, and also in tumours at all clinical stages of progression.

Introduction

Two observations suggest that an anti-oncogene concerned with the regulation of colorectal mucosal growth is situated on the long arm of chromosome 5. First, familial adenomatous polyposis—a Mendelian dominant condition characterised by development of large numbers of colorectal adenomas—is transmitted by a gene (*APC*) mapping within 5q21-22 (Bodmer *et al.*, 1987; Leppert *et al.*, 1987). Second, hemizygous loss of at least part of 5q has been reported in 19–36% of sporadic colorectal carcinomas (Solomon *et al.*, 1987; Okamoto *et al.*, 1988; van den Broek *et al.*, 1988; Vogelstein *et al.*, 1988; Law *et al.*, 1988; Rees *et al.*, 1989). There is, however, little information on the status of *APC* itself in these tumours. At other, better-defined anti-oncogene loci, such as the genes conferring susceptibility to Wilms' tumour and retinoblastoma, acquired allele loss within the tumours commonly involves whole chromosomes (Dryja *et al.*, 1984; Fearon *et al.*, 1984). Such losses are probably the result of mitotic non-disjunction. In contrast, recent studies in human colorectal and breast carcinoma have revealed a high frequency of acquired homozygosity of 17p alleles (Monpezat *et al.*, 1988; Vogelstein *et al.*, 1988; MacKay *et al.*, 1988), but here the loss is commonly achieved through partial arm deletion or mitotic recombination, requiring an intrachromosomal breakage event. In this paper we examine the status of *APC* in a large series of sporadic colorectal carcinomas. Using DNA probes homologous to polymorphic sequences flanking and close to *APC* ('closely-flanking markers'), we show that *APC* alleles are lost in a much higher proportion of tumours that hitherto recognised. Whole chromosome loss is rare, the majority of lesions resulting from intrachromosomal breakage (interstitial deletions, mitotic recombinations or partial arm losses). The results have interesting implications on the status of *APC* in colorectal carcinogenesis, and its susceptibility to nearby chromosome breakage events.

Received 22 May 1989; accepted in revised form 21 June 1989

Results

Loss of APC *in sporadic tumours*

There were 119 tumours, confirmed as carcinomas by histology in all but 2 cases, the exceptions both being large villous adenomas. Pairs of closely-flanking markers lying centromeric and telomeric to *APC* within 5q21-22 were used to detect tumours exhibiting hemizygous loss of polymorphic restriction fragment alleles. To establish the physical extent of allele loss, tumour DNA was analysed with probes mapping to regions spanning chromosome 5 at 5p, 5q12-14 (close to the centromere) and 5q35 (telomeric). Details of the probes used are given in Table 1 and examples of allele loss demonstrated with each are shown in Figure 1. The

Table 1 Probes used in analysis of chromosome 5 alleles

Probe	*Specification of target sequence*	*Enzyme*	*Alleles kb*	*Ref*
L1.4	D5 S4	EcoR1	0.7, 0.6	1, 2
MLZ 7.5	—	EcoR1	2.9, 2.2, 3.2*	1
105-153 Ra	D5S39	MspI	8.0, 5.0	1, 2
105–599 Ha	D5S76	TaqI	17.0, 14.0, 10.0	1, 2
105-789 Rb	D5S78	MspI	2.3, 1.8	1, 2
π 227	D5S37	BclI	3.0, 1.8, 1.2	2, 3, 4, 5, 6
C11P11	D5S710	TaqI	4.4, 3.9, 5.0*	3, 7, 8
YN5.48	D5S81	MspI	9.0, 8.0	3, 8
MC5.61	D5S84	MspI	5.5, 5.0	3, 8
λ MS8	D5S43	HinfI	2.4–9.5	2, 9

* Constant allele

References: 1. Leppert *et al.* (1987)
2. Olaison *et al.* (1987)
3. Nakamura *et al.* (1988)
4. Dunlop *et al.* (1989)
5. Meera Khan *et al.* (1987)
6. Stewart *et al.* (1987)
7. Bodmer *et al.* (1987)
8. Wasmuth & Ferrell (1988)
9. Solomon *et al.* (1987)

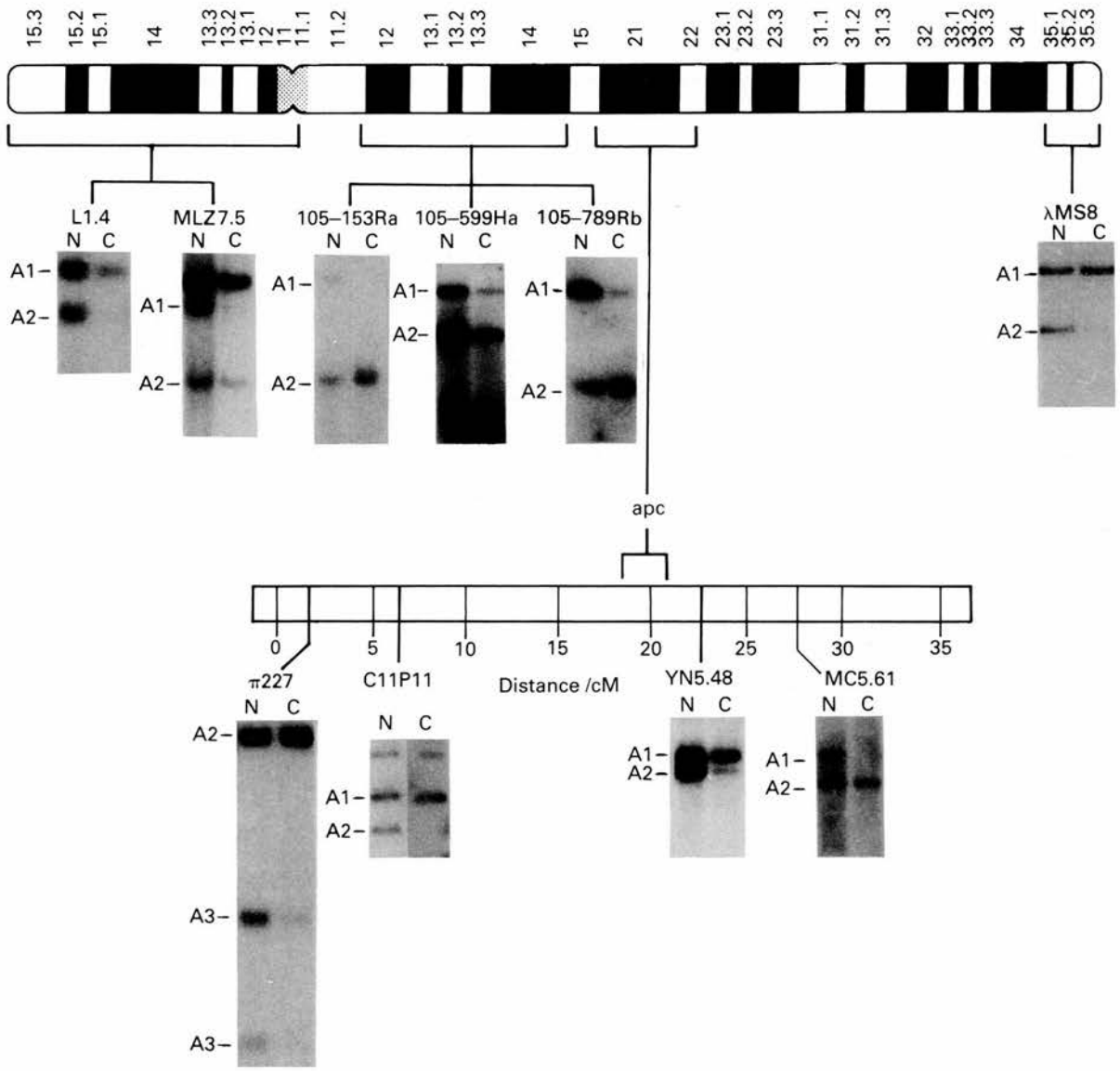

Figure 1 Location of polymorphic sequences in chromosome 5 detected by the ten probes used in this study (Table 1). The genetic distances between the *APC* markers are given in centimorgans (1 cM equivalent to 1 megabase (1 mb) in humans). Distances are reproduced from a single early study (Nakamura *et al.*, 1988) for the sake of consistency. More recent data, however (M.G. Dunlop, Y. Nakamura, unpublished), indicates that the distance between π 227 and C11P11 may be as little as 2 cM and between C11P11 and YN5.48, approximately 10 cM

overall frequency of loss observed for any 5q-linked allele was 48% (Table 2A), substantially higher than that quoted in previous studies. A similar high frequency (45%) was found when analysis was restricted to tumours showing loss of alleles in 5q21-22 (Table 2B). Of the 65 tumours informative with closely-flanking markers on both sides of *APC*, 35 (54%) showed loss of a flanking allele (Table 2C), and in the great majority of these (24/35; 69%) the loss was unilateral. The unilateral loss of closely-flanking markers occurred centromerically and telomerically to *APC* in equal proportions indicating the existence of a cluster of breakpoints within a 10–15 mb region either side of *APC*.

Nature of defects in chromosome 5

Information from tumours mapped with closely-flanking markers, when combined with data from markers mapping to outlying regions of chromosome 5, allowed a physical interpretation of 5q defects (Table 2D). Of the 32 tumours exhibiting loss of one or more of the alleles within 5q21-22, the great majority remained heterozygous for one other chromosome 5 allele at least. This demonstrates that loss of the whole of chromosome 5 plays a minor role in generating *APC* allele loss in sporadic colorectal cancer. The commonest lesion (20/32; 63%) was interpreted as interstitial deletion since the tumours remained heterozygous for markers on both sides of the region showing allele loss. A qualitative depiction of the data (Figure 2) shows the large contribution of breakpoints close to *APC* in these deletions, of which the smallest observed was about 10–15 mb long (the distance between YN5.48 and C11P11; Y. Nakamura unpublished data). The remaining class of chromosome lesions included fewer breaks close to *APC* but showed a striking bias towards the loss of markers telomeric to *APC*. Presumably these represent mitotic recombinations or partial arm losses, which are not distinguished from each other by this type of analysis. The mapping of these allele losses reinforces the view that a specific region, inclusive of the *APC*

Table 2 Analysis of allelic losses in 119 colorectal tumours

		Percentage:	
	No of cases	*Of total*	*Of cases with allele loss*
A. Informative within 5q	117	(100%)	
Allele loss within 5q	56	(48%)	
B. Informative at 5q.21-22	107	(100%)	
Allele loss at 5q.21-22	56	(48%)	
C. Informative at closely-flanking loci on both sides of *fap*	65	(100%)	
Loss of at least one flanking allele	35	(54%)	(100%)
Loss of one flanking allele	24		(59%)
Loss of both flanking alleles	11		(31%)
D. Permitting physical interpretation of 5q defect	68	(100%)	
Total with 5q.21-22 allele loss	32	(48%)	(100%)
Interstitial deletion	20		(63%)
Partial arm loss/mitotic recombination	11		(34%)
Whole chromosome loss	1		(3%)

gene, is commonly deleted in sporadic colorectal carcinoma. Of 36 carcinomas showing retention of a pair of closely-flanking markers in 5q21-22 only 1 showed the loss of an outlying 5q allele.

Loss of APC *and tumour progression*

In an attempt to ascertain the phase of tumour evolution at which *APC* alleles are most commonly lost, we studied the frequency of loss in tumours that differed in stage of progression, or in DNA ploidy. There was no correlation ($P > 0.05$) between the degree or occurrence of DNA aneuploidy (as determined by flow cytometry) and *APC* loss (Table 3). Nor did loss of *APC*-flanking markers correlate with Dukes' staging of the extent of tumour spread. It thus appears that the loss of *APC* takes place independently of (and probably before) the emergence of features determining the progression of the carcinoma. This agrees with the observation of 5q21-22 allele loss in both of the villous adenomas in this study. Such tumours do not show infiltration or metastasis but carry a high probability of progressing to carcinoma.

Discussion

The high frequency of 5q21-22 loss recorded in this study is due entirely to the use of closely-flanking markers on both sides of *APC*. Previous studies using unilateral or distant markers, (Solomon *et al.*, 1987; Okamoto *et al.*, 1988; van den Broek *et al.*, 1988; Vogelstein *et al.*, 1988; Law *et al.*, 1988; Rees *et al.*, 1989) suggested lower frequencies of loss, because they failed to detect small deletions including *APC*. This implies that as one approaches the *APC* locus the frequency of observed allele loss may increase still further and indeed that many of the important lesions may be within *APC* itself. A similar situation at 13q14 occurred in sporadic retinoblastoma, where small deletions of the Rb-1 gene, not detected cytogenetically, were revealed by the use of intra-gene molecular probes (T'Ang *et al.*, 1988) and closely-flanking markers (Dryja *et al.*, 1986).

Table 3 Prognostic indicators and 5q21-22 status

	*5q21-22 status**	
Tumour status	*Retained*	*Lost*
Villous adenoma	0	2 (100%)
Carcinoma — Dukes A	1	8 (89%)
— Dukes B	16	20 (56%)
— Dukes C	14	19 (58%)
DNA diploid	2	6 (75%)
DNA aneuploid	8	11 (58%)

* Tumours were scored as 5q21-22 retained only if an allele within q21-22 on *each* side of the *APC* locus retained heterozygosity. Loss of a 5q21-22 allele on either or both sides of *APC* scored as loss

The low frequency of whole chromosome 5 loss argues against mitotic non-disjunction as a major mechanism for the acquired loss of *APC* in tumours. Many reported karyotypes in colorectal cancer cells are hypotriploid or hypotetrapoid, (Reichmann *et al.*, 1981; Petersen & Friedrich, 1986) consistent with origin through non-disjunctive events from a tetraploid parent (Nowell, 1976). The majority of human colorectal cancers do show DNA aneuploidy in this range, as determined by flow cytometry. As the presence of aneuploidy correlates with poor prognosis (Armitage *et al.*, 1985; Quirke *et al.*, 1987), it is tempting to suggest that this mode of chromosome loss contributes to the genesis of homozygosity at anti-oncogene loci during tumour progression (Wyllie, 1989). Our data, however, make it clear that whole chromosome non-disjunction is a rare cause of acquired homozygosity at the *APC* locus. Rather, it appears that the *APC* gene is lost early in the evolution of colorectal tumours. This is further supported by the association of presumptive inherited molecular defects within the *APC* gene in familial adenomatous polyposis where the genetic abnormality is primarily responsible for formation of multiple adenomas. Loss of *APC* early in the adenoma-carcinoma sequence is also suggested by the work of other groups (Remvikos *et al.*, 1988; Vogelstein *et al.*, 1988).

Cytogenetic findings in sporadic colorectal carcinoma (Muleris *et al.*, 1985; Ferti *et al.*, 1988) confirm the present findings of a low frequency of whole chromosome 5 loss. However the fact that such lesions (and the commoner 5q arm losses) are tolerated shows that survival is possible in cells which are hemizygous for genes on 5q outside *APC*. Thus the observed clustering of breakpoints around *APC* is unlikely to be merely the consequence of positive selection for lesions which delete *APC* but conserve important outlying genes.

This clustering of breakpoints is not predicted by a simple interpretation of the anti-oncogene hypothesis of Knudson (1983). Other putative anti-oncogenes involved in colorectal carcinoma, such as those on 17p 12–12.3 (Lothe *et al.*, 1988; Baker *et al.*, 1989), and 18q (Monpezat *et al.*, 1988; Vogelstein *et al.*, 1988), have not yet been analysed in sufficient detail to measure the contribution of different types of chromosomal lesions to the allele loss. Clustered breakpoints are not observed, however, in the somatic loss of anti-oncogenes at 13q14 in retinoblastoma (Dryja *et al.*, 1984) or 11p13 in Wilms' tumour (Fearon *et al.*, 1984), where whole chromosome loss, mitotic recombination and partial chromosome arm loss played a greater role than in the present study. The finding of molecular

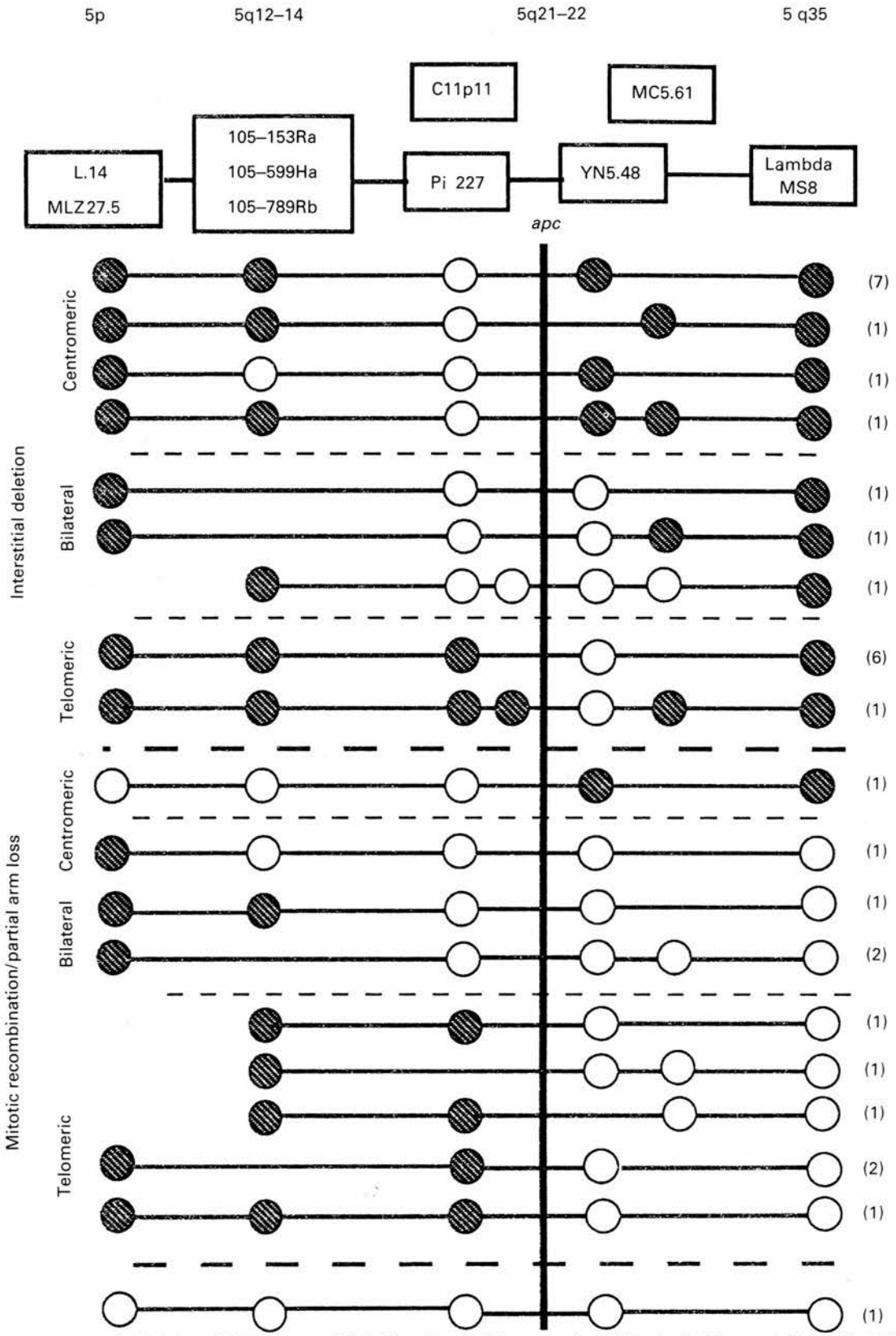

Figure 2 Patterns of allelic loss of chromosome 5 linked markers in 32 tumours in which physical interpretation of the defect was possible. Thirty were carcinomas, two villous adenomas. Uppermost, schematically shown, is the location of polymorphic DNA probes on chromosome 5. Aligned below is the allelic loss (open circle) or retention (shaded circle) revealed by these markers. The number of tumours exhibiting a particular pattern of loss is indicated in brackets on the right-hand side and similar patterns are grouped together as interstitial deletions (centromeric, bilateral, telomeric) or mitotic recombinations/partial arm loss (centromeric or telomeric), involving *APC*

lesions grouped around the *APC* locus in a high proportion of sporadic colorectal carcinomas suggests that this chromosomal region may possess some innate feature predisposing it to breakage and recombination events. This, as well as the putative altered properties of the *APC* gene product(s), may be a factor influencing the contribution of the locus to the progression of colorectal cancer. A constitutive 'fragile' site for chromatin

reakage has been identified in the vicinity of 5q21.2 Yunis *et al.*, 1987), although its precise relationship to *APC* is unknown. More detailed mapping of the region n and around *APC* may clarify the mechanisms under- ying the genetic changes observed in these common uman tumours.

Materials and methods

Clinical material

One hundred and nineteen tumours from 117 patients under- oing operation for colorectal carcinoma were sampled. The olons were placed on ice immediately after removal, and vithin 20 min were opened and rinsed in ice-cold phosphate- uffered saline, supplemented to 0.02% (w/v) with EDTA. mall portions of tumour, and distant apparently normal ucosa were snap frozen in liquid nitrogen. Immediately djacent tissue was fixed in neutral buffered formalin or PLPD (Holgate *et al.*, 1986) for histology. The remainder of he specimen was examined after fixation for depth of tumour nvasion and lymph node infiltration to permit staging in the Dukes' classification. In all there were 117 carcinomas and wo large villous adenomas.

DNA analysis

DNA of high molecular weight was prepared from tissue sing a method based on SDS-lysis and proteinase K- igestion as described by Goelz *et al.* (1985), digested (10 μg) vith the appropriate restriction enzyme and electrophoresed hrough 0.8% (w/v) agarose gel DNA was transferred on to ylon membranes (Southern, 1975) in 0.5 M NaOH, 1.5 M NaCl. Prehybridization was performed for at least four hours t 65°C in 10% (w/v) dextran sulphate, 1% (w/v) SDS, 0.9 M NaCl–0.09 M sodium citrate, 100 μg ml^{-1} denatured salmon perm DNA. Hybridization was at 65°C for 16–24 h in the same solution, together with salmon sperm DNA at 250 μg ml^{-1} and probe labelled with α-[^{32}P]dCTP (specific activity 10^9 cpm μg^{-1}) by the random priming method (Feinberg & Vogelstein, 1983, 1984). Membranes were washed as described (Nakamura *et al.*, 1988) and exposed at −70°C. Hybridization conditions were altered for probes with high levels of non-specific binding; human placental DNA was used instead of salmon sperm DNA, and the probe pre-incubated with the hybridization solution at 65°C for one hour before addition to the membrane.

For each probe the target locus specification, preferred restriction enzyme, and allele sizes are given in Table 1. Allele losses were accepted only if the differences in band intensity on the autoradiogram were obvious.

Flow cytometry

Small portions of frozen tissue, taken adjacent to the sites sampled for histology and DNA analysis, were used to prepare suspensions of single nuclei by the detergent-trypsin procedure of Vindeløv *et al.* (1983). Chicken erythrocytes were mixed with the sample at the beginning of the preparation procedure to serve as an internal DNA ploidy standard. The DNA content of these cells is about 35% of that of normal human diploid cells. Nuclei stained with 0.62 M propidium iodide were analysed in a Coulter Epics CS flow cytometer at an excitation wavelength of 488 nm. Coefficients of variation of G_0/G_1 peaks seldom exceeded 3%. Aneuploidy was considered present when two distinct G_0/G_1 peaks were visible.

Acknowledgements

This work was supported by a grant to CCB and AHW from the Cancer Research Campaign. MGD is a MRC Training Fellow. We thank the clinical staff of surgical wards in the Royal Infirmary and Western General Hospital, Edinburgh, for their commitment to this project, and M. Somerville, R.M. Hogg and D. Bishop for technical assistance.

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experimental group compared with only 11% of the conventionally treated group had a remission (defined as at least 3 months of good control on the same diet and oral agents that had previously been shown to be ineffective). At the time of their report three of the remissions in the experimental group had lasted up to a year and two of these were continuing. Enthusiasm for this approach waned as other workers were unable to produce prolonged remissions. A Danish group[12] achieved tight control with multiple daily injections of insulin and showed improvement in beta cell function compared with a conventionally treated group, but by 90 days the difference was no longer apparent. Perlman[13] used insulin delivery by a portable intravenous pump for 28–62 days to produce tight control; he also showed higher C peptide levels by comparison with a conventionally treated control group initially but by 4 months there were no differences in C peptide nor in glycosylated haemoglobin levels between the two groups. Renewed interest in this area has been stimulated by a report from Shah and colleagues.[14] In a randomised trial, twelve patients with newly diagnosed IDDM (mean age 13·2 years) were treated for 2 weeks with an artificial pancreas ('Biostator') to maintain normoglycaemia. A control group of fourteen patients of a similar age were treated with twice daily isophane insulin, adjusted to maintain a blood glucose below 7·8 mmol/l before meals and at 11·1 mmol/l an hour after meals. The biostator group were subsequently managed on the same regimen as the controls. Beta cell function was assessed at intervals for a year. At 1 year there were significantly higher C peptide levels and lower glycosylated haemoglobin levels in the experimental group.

It is not clear why different studies have shown that the benefits of tight control last for variable lengths of time. The degree of tight control achieved and its duration may be important. In Shah's study, the control group were especially poorly controlled after a year, making it difficult to distinguish cause from effect. Were they poorly controlled with a reduced capacity to produce endogenous insulin because they were not treated initially with a biostator or was it because they had received insufficient exogenous insulin for several months?

Treatment with an artificial pancreas for 2 weeks immediately after diagnosis would be very difficult to implement in clinical practice since very few centres have access to a biostator. The machines are very expensive and need specialised staff to use them. The patient has to be admitted to hospital and remain attached to the machine continuously by intravenous cannulae. Even if time and money were no object, this experience might well prove to be very traumatic. Psychological disturbances are common in patients with IDDM,[15,16] and adolescence is especially fraught with difficulties.[17] Behaviour problems can cause even more disruption of diabetic control than can reductions in C peptide levels.[18] Adolescents often discuss how upset they were at the time of diagnosis. Many clinicians feel it is important to reassure them that they can continue to live their lives without too many restrictions, so it is usual to treat them as outpatients to minimise interference with normal activities.

It seems sensible to treat patients with newly diagnosed diabetes with conventional methods to achieve as tight control as is possible without undue risk of hypoglycaemia. Clinical diabetologists will require far more evidence to convince them of the value of imposing upon young, vulnerable individuals the rigours of 2 weeks' attachment to an artificial pancreas.

Colon Cancer: Molecular Analysis Marches On

COLORECTAL cancer is common in Western societies. Both environmental (mainly dietary) and genetic factors are implicated in its aetiology, while the progression through hyperplasia, adenomatous change, pre-invasive, invasive, and finally metastatic carcinoma is more clearcut in this disease than in many other malignancies.[1,2] Thus colorectal cancer has become an obvious focus for examination of the molecular events underlying the evolution of cancer in general.

An important lead was provided by studies of familial adenomatous polyposis (FAP), including Gardner's syndrome.[3] This condition is inherited as an autosomal dominant and affects about one in ten thousand people. In affected individuals during early adulthood, hundreds or thousands of adenomas develop throughout the gastrointestinal tract but especially in the colon. These tumours progress almost invariably to carcinoma unless the large bowel is removed. Linkage studies in families mapped the gene responsible (*apc*) to the long arm of chromosome 5 within bands q21–22.[4,5] It has subsequently been

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shown that at least a proportion of sporadic colon cancers carry a deletion that appears to involve the same *apc* gene.[6-10] This gene therefore has strong credentials as a tumour suppressor by analogy with retinoblastoma, in which familial and sporadic tumours appear to have molecular lesions involving the same gene (*Rb*)[11] on chromosome 13q14. As predicted by De Mars[12] and presented as a detailed hypothesis by Knudson,[13] at least two mutations are required before a tumour can develop. However, it is unclear whether one or both copies of *apc* must be inactivated before a tumour will develop in the colon. Until the gene itself has been identified and cloned, point mutations or other very subtle changes that may affect its function cannot be detected. Meanwhile, reliance is placed on flanking DNA markers, which will be absent only when a substantial segment of the chromosome is deleted. Within that segment there may be one or more genes that are essential (at least in single copy form) to the survival of the cell, so large deletions will always be heterozygous. One can only speculate about the integrity or otherwise of the *apc* gene on an apparently intact chromosome 5, but it is relevant that in FAP, in which one copy of the gene is presumed already to be abnormal, molecular analysis of colonic adenomas seldom yields any evidence that the other copy has been deleted.[6-9,14,15]

Knudson's hypothesis requires that one genetic lesion should not be sufficient for tumorigenesis but is entirely compatible with second and subsequent mutations affecting different genes. Karyotypic analysis of colon cancers has shown that the long arms of chromosomes 5[16,17] and 18[18] and the short arm of chromosome 17[17,18] are consistently involved in non-random losses, thereby identifying potential sites for other tumour suppressor genes that might act in concert with *apc*. This evidence has been corroborated at the molecular level by the finding of frequent allele losses at loci on 18q and, most strikingly, on 17p.[9,10,18-22] Over 70% of sporadic colon cancers have lost a region of chromosome 17 short arm that includes a gene (p53) thought to be implicated in the neoplastic process.[23] Sequence data have documented point mutations within a highly conserved region of the p53 gene on the remaining chromosome 17 in tumours shown to have lost chromosome 17 DNA markers.[21] However, specific point mutations within the p53 sequence, far from inactivating it, can convert it to a cellular transforming oncogene,[23] and so the distinction between oncogene (dominant) and tumour suppressor gene (recessive) is now somewhat blurred.

Other changes in the DNA of colon tumours that are implicated in carcinogenic progression include activation of the Kirsten *ras* oncogene,[9,24,25] hypomethylation of cytidine residues[26] (associated with abnormal levels of gene activation), and aneuploidy.[27] It seems very likely that a single genetic change contributing to the development of a tumour may enhance the risk of second and subsequent events by inducing hyperplasia (thereby increasing the population of cells at risk), by compromising the stability of the rest of the genome, or by some combination of these and other mechanisms. At least some of the events may be common to many different types of tumour and it is not yet clear how many of them are required, or in what permutations, to drive a cell to a given degree of malignancy. There is, as might be expected, a correlation between tumour stage and the total number of identifiable genetic lesions in colon cancers,[10] but the order in which the genetic changes accumulate may be flexible.[9] Some data suggest that loss of the *apc* locus tends to be an early event whereas deletions on 17p and 18q come later.[9]

These latest findings in colorectal cancer illustrate very clearly how molecular genetics is transforming our knowledge of the fundamental basis of malignancy. Nevertheless, it is important not to concentrate on the cell nucleus and its contents to the exclusion of other elements in the complex equation. Even in FAP, in which a single gene holds centre stage, the observation that established adenomas regress when the bowel contents are altered by subtotal colectomy with ileorectal anastomosis[28] is a

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salutary reminder that environmental influences must play a part in determining the outcome of genetic events.

SURVEILLANCE CULTURES IN NEUTROPENIA

PROFOUND neutropenia (white cell count $< 0{\cdot}5 \times 10^9$/l) is associated with a greatly increased risk of infection, which may develop rapidly and lead to death within hours.[1,2] Reliable prediction of the identity and antibiotic susceptibility of the infecting organism would help management of neutropenic patients considerably. About half of all proven bacteraemic episodes arise from the patient's own microbial flora;[3] most of the remainder stem from organisms acquired in hospital from sources such as hands of attendant staff[4] and uncooked food.[5] Close monitoring of microbial flora of individual neutropenic patients, by regular sampling of several sites, could theoretically detect likely pathogens before a bacteraemic episode occurred and so initial antibiotic treatment could be tailored to cover these organisms. This happy outcome has not always been borne out in practice. Whilst surveillance cultures are undoubtedly valuable for elucidation of epidemiological patterns and design of infection prevention regimens, their routine use as a predictor of infection remains controversial.

Monitoring of several sites—eg, nose, axilla, gingiva, and rectum—as done by earlier researchers[6] is very time consuming and costly for the laboratory. Moreover, unless a patient is receiving oral, nonabsorbable antibiotics or selective gut decontamination (SGD) regimens, his microbial flora, although not hospital-acquired, will number dozens of bacterial and fungal species. Routine selection and characterisation of any but those few organisms most commonly implicated in infection—eg, *Pseudomonas aeruginosa*—is therefore impractical. Information gained in this way is of limited value, because almost any resident microorganism may initiate infection in patients with neutropenia, and a small but significant number of bacteraemias are caused by organisms not previously detected by the most rigorous surveillance.[7]

Culture results themselves depend on several variables—eg, the quality of the specimen and of laboratory processing. The single most useful specimen for the detection of gram-negative organisms is faecal material.[8] In general, more useful bacteriological information is obtained in patients undergoing SGD, in whom the numbers of microorganisms are greatly reduced. A very high positive predictive value for development of infection has only been obtained with the isolation of *Aspergillus flavus* from nasal swabs: in one study, invasive aspergillosis developed in 10 of 11 patients with positive cultures.[9] As might be expected, negative cultures have a consistently high negative predictive value.

Wider use of SGD and of central venous catheters has led to a change in the spectrum of bacteria causing infection in neutropenia and consequently to reappraisal of the role of surveillance cultures. Gram-positive organisms such as coagulase-negative staphylococci and diphtheroids now occur more frequently. In one study, gram-positives accounted for 20% of bacteraemias in the 5-year period before introduction of SGD, but for 62% in the following 5 years.[10] Central venous catheters are an important source of infection, bacteria being introduced during catheter manipulation, tracking along the catheter surface, or lodging on the catheter via haematogenous spread. Studies of the value of surveillance cultures of the skin at the catheter entry site and catheter hub have given conflicting results. Some workers have found them useful,[11] but others have found that an initial site of infection could be identified in only half of all gram-positive septicaemias.[12] The importance of coagulase-negative staphylococci is very difficult to assess; although these skin commensals are the most common colonisers of central venous cannulae, they are also the most common contaminating organisms.[13] Most gram-positive infections are caused by organisms of low virulence, which tend to cause a low-grade illness, giving clinicians a little extra time if the bacterium isolated on culture is found to be resistant to initial antibiotic therapy.

Some researchers are convinced that surveillance cultures aid the earlier, more targeted, initiation of antimicrobial therapy[8,14] whereas others think them "costly, insensitive and unlikely to influence antimicrobial management".[15] The cost of surveillance cultures is undisputed, especially since no infecting organism is identified in over 80% of all febrile episodes in neutropenic patients.[16] In light of the published data, it would be rash to commit a patient to narrow-spectrum antimicrobial therapy purely on results of such cultures. However, in certain circumstances—eg, heavy colonisation with a particularly invasive organism or nasal colonisation with *Aspergillus flavus*—additional, sometimes lifesaving therapy might be started earlier.

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Inheritance of colorectal cancer susceptibility

Although dietary factors are clearly implicated in the aetiology of large bowel malignancy, there is substantial evidence to suggest that susceptibility to colorectal cancer is inherited. Many studies[1,2,3] have shown familial clustering of benign and malignant colorectal neoplasms which is not restricted to the syndrome of hereditary non-polyposis colorectal cancer. Lovett[1] noted a slight excess of colorectal cancer in the spouses of affected individuals but subsequently it has been shown that familial clustering of cases cannot be accounted for by the simple hypothesis of common exposure to environmental carcinogens[3,4]. The data support the existence of a gene defect which results in colorectal adenomas and carcinomas with a population frequency of 19 per cent and which is transmitted in a Mendelian dominant mode[3]. In addition, it appears that very few cancers occur in patients who have not inherited a predisposition to large bowel neoplasia. If true, this has resounding implications for screening and early detection of premalignant and malignant colorectal neoplasms.

If there is a heritable colorectal cancer gene, the techniques of modern molecular biology may eventually be able to provide a simple screening procedure involving only a blood test. This would identify those with a gene defect predisposing the large bowel epithelium to the effects of faecal carcinogens. Dietary manipulation seems unlikely to be practical and so such individuals could be enrolled in intensive clinical screening programmes. Such a screening protocol seems eminently desirable for its efficiency and presumed sensitivity. However molecular biologists need clinical data which not only confirm that a colorectal cancer gene does actually exist, but also identify those patients who have inherited it. Clearly not all individuals with the gene defect will develop colorectal cancer and so there is a pressing need for a full description of the 'colorectal cancer gene' phenotype. There are already some pointers as to what such a phenotype might be.

Skin tags are more common in patients with colorectal adenomas than in the general population[5]. It seems unlikely that gut carcinogens should also have an effect on skin tag formation and so such patients may have inherited a defect in a gene with pleiotropic effects involving skin and colonic epithelium. Mandibular osteomas are common in patients with familial adenomatous polyposis (FAP) and these lesions also occur with a higher frequency in patients with apparently non-familial colorectal cancer than in a control population[6]. This suggests that the 'colorectal cancer gene' may actually be the gene for FAP (the so-called *apc* gene) but perhaps carrying a different class of mutation within it. There is molecular genetic evidence to support this hypothesis since *apc* gene deletion has been shown to occur in a high proportion of colorectal carcinomas[7]. If the *apc* gene on chromosome 5 is involved in the inheritance of susceptibility to colorectal cancer then other features of FAP may be prevalent in colorectal cancer patients.

In this issue, Iwama and colleagues[8] examine the association of congenital hypertrophy of the retinal pigment epithelium (CHRPE) and FAP. They, and others[9], have demonstrated the prevalence of this phenotypic marker in FAP patients. If there is a class of inherited *apc* gene mutation which predisposes to colorectal cancer, then CHRPE lesions may occur with undue frequency in patients with large bowel malignancy.

It is clear that the diagnostic work-up of patients with colorectal neoplasms should include a careful family history but studies to delineate the clinical phenotype of the putative colorectal cancer gene are now also required.

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Metabolic effects of cancer

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The potential causes of deranged metabolism in cancer are discussed with emphasis on changes in energy metabolism of glucose, fat and protein. The implications of these changes for the treatment of cachexia are then considered

Keywords: Cancer, cachexia, metabolism, glucose, fat, protein, parenteral nutrition

Cachexia is commonly the cause of death in cases of advanced malignancy[1], and cancer patients who have lost a significant percentage of their body-weight before surgical treatment are subject to a much greater risk of postoperative mortality and morbidity[2–4]. There is no doubt that reduced oral intake resulting from anorexia or obstruction of the gastrointestinal tract plays a very significant role in the development of the cancer cachexia syndrome. However, whereas the metabolic response to uncomplicated starvation acts to limit the consumption of host reserves, in the cachectic cancer patient there is often an accelerated mobilization and oxidation of energy substrates and loss of nitrogen[5–7]. These changes are a consequence of alterations in intermediary metabolism associated with cancer[8].

Understanding the metabolic response to cancer has become increasingly important over the last two decades with the introduction of effective and safe parenteral nutrition techniques[9]. It is now possible to provide sufficient calories and nitrogen to all cancer patients, but the metabolic milieu associated with advanced cancer may retard the restoration of lean body mass[10]. In the following review the manner in which malignant tumours affect host metabolism will be presented, and the effectiveness of the available therapeutic options will be discussed.

One consistent feature of data from metabolic studies in cancer patients is the range of response between individuals, even when comparing those with the same diagnosis and stage of disease[11]. The interpretative difficulties are compounded by many reports comparing small heterogeneous groups of cancer patients with equally small groups of controls, which may well be poorly matched for age or weight loss. To overcome some of these problems laboratory models have been developed in which malignant cells of identical genotype are transplanted into genetically uniform animals[12]. However, the growth dynamics and tumour-to-host weight ratios frequently do not resemble those observed in patients, and this review will present mainly data from patient studies.

Changes in energy metabolism

The hypothesis that tumour bearing increases energy expenditure and results in a cumulative negative energy balance and progressive weight loss has been exhaustively investigated, and there is now a substantial body of supportive evidence. Bozzetti *et al.*[13] studied a heterogeneous group of patients with advanced tumours and found a highly significant correlation between the resting metabolic expenditure (RME) and the magnitude of weight loss, and other groups of researchers have similarly found elevated RMEs in patients with cancer cachexia[14–18]. There are a few anecdotal reports of cases in which successful antineoplastic therapy has reduced energy expenditure in hypermetabolic patients[14,18], suggesting that the presence of the tumour itself is capable of elevating the RME. However, hypermetabolism is not an invariable finding in cancer patients who have lost weight, with large series having been reported recently which have failed to demonstrate a significant increase in the resting metabolic rate of cachectic cancer patients when compared with patients with weight loss of a similar magnitude due to benign disease or with weight-stable cancer patients[19,20]. In a series consisting of 200 patients with a variety of tumour types 29 per cent had a resting metabolic expenditure that was 10 per cent higher than that predicted by the Harris–Benedict equation, 31 per cent were found to be hypometabolic using the same criterion and no relationship was demonstrated between RME and weight loss or tumour burden[21].

Although some of the disparity in the findings of these studies is no doubt a reflection of differences in experimental material and methodology, it is likely that they are reflecting a true heterogeneity of response to the tumour bearing state. It is now clear that cancers arising from certain tissues, such as sarcomas[22], leukaemias[23] and bronchial carcinomas[24], frequently provoke a hypermetabolic response, whereas patients with pancreatic and hepatobiliary tumours tend to be hypometabolic[25].

Many cancer patients with advanced disease have a reduced caloric intake. In normal people or in patients with benign disease, semistarvation is attended by a reduction in RME[26,27] so in an undernourished cancer patient even a normal metabolic rate represents a failure of this adaptive response[14,28].

The mechanism by which malignant tissue alters the energy expenditure of the host is not clear. It is unlikely that increased energy consumption by the tumour itself is responsible in human tumours as it is rare for tumours to account for more than 5 per cent of body weight[13]. More plausible is the hypothesis that mediators are released by some cancers which alter host metabolism[29,30], and some of the changes that may occur are discussed in subsequent sections.

Changes in glucose metabolism

There are many reports describing an increased rate of endogenous glucose production in cancer patients[11,22,23,31–33] (*Figure 1*), and considerable research effort has been directed towards determining the mechanism and significance of this occurrence. It is clear that the magnitude of the increase in glucose turnover is influenced by tumour stage[6,35] and histology, and that it is associated with cancer cachexia[36]. In this section, some of the observations made in cancer patients of changes in glucose metabolism will be summarized and the implications that these have on energy balance will be discussed.

Gluconeogenesis

Shaw and Wolfe[6] have defined glucose kinetics in a group of

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kinesin could serve primarily to establish that array but not to maintain it. The behaviour of lysosomes observed in living cells indicates, however, that the lysosome distribution is maintained by a continuous balance of outward and inward vesicle movements. We therefore suggest that kinesin ATPase has a role in the formation and maintenance of the lysosomal compartment in the form of an extended tubulovesicular array in macrophages. On the basis of immunolocalization[5,17] and *in vitro* motility studies[3,4,13,18] this probably involves kinesin molecules anchored to the organelle surface which undergo ATP-dependent interaction with microtubules to produce centrifugally directed translocating forces. □

Received 15 January; accepted 15 June 1990.

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ACKNOWLEDGEMENTS. We thank H. McNamee for assistance with preliminary experiments, J. M. Scholey and M. P. Sheetz for advice on *in vitro* motility assays, and J. M. Scholey for the gift of SUK4 hybridoma cells. This work was supported by the NIH.

Telomere reduction in human colorectal carcinoma and with ageing

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WE have hypothesized that end-to-end chromosome fusions observed in some tumours could play a part in genetic instability associated with tumorigenesis and that fusion may result from the loss of the long stretches of G-rich repeats found at the ends of all linear chromosomes[1]. We therefore asked whether there is telomere loss or reduction in common tumours. Here we show that in most of the colorectal carcinomas that we analysed, there is a reduction in the length of telomere repeat arrays relative to the normal colonic mucosa from the same patient. We speculate on the consequences of this loss for tumorigenesis. We also show that the telomere arrays are much smaller in colonic mucosa and blood than in fetal tissue and sperm, and that there is a reduction in average telomere length with age in blood and colon mucosa. We propose that the telomerase[2–4] is inactive in somatic tissues, and that telomere length is an indicator of the number of cell divisions that it has taken to form a particular tissue and possibly to generate tumours.

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The termini of human chromosomes, like those of other eukaryotes, comprise stretches of G-rich repeats[5–10] which are of ~10 kilobases (kb) and 15 kb in blood and sperm respectively[5,7,10,11]. When DNA is digested with a frequently cutting restriction enzyme such as *Hin*fI or *Alu*I, which will not cut within the repeat, subsequent probing with a labelled oligonucleotide for $(TTAGGG)_n$ will reveal the size of the terminal repeat array (TRA). Figure 1*a* shows the hybridization of a $(TTAGGG)_4$ probe to *Hin*fI-digested DNA from matched normal colonic mucosa and colon carcinoma samples from seven individuals. There is some variation in the average length of the TRA between normal mucosae from different individuals but not much difference in signal strength. In all the carcinomas there is a reduction in length of the repeat stretch relative to the respective normal mucosa and in some cases there is a marked reduction in the signal intensity.

We repeated the experiment with the restriction enzyme *Alu*I and obtained a very similar result to that obtained with *Hin*fI

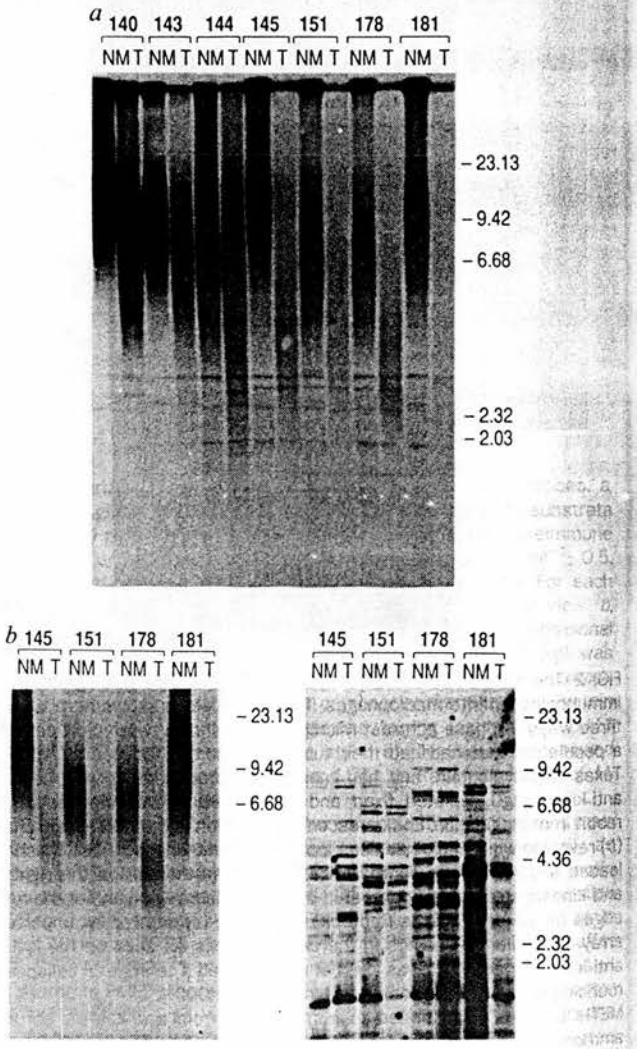
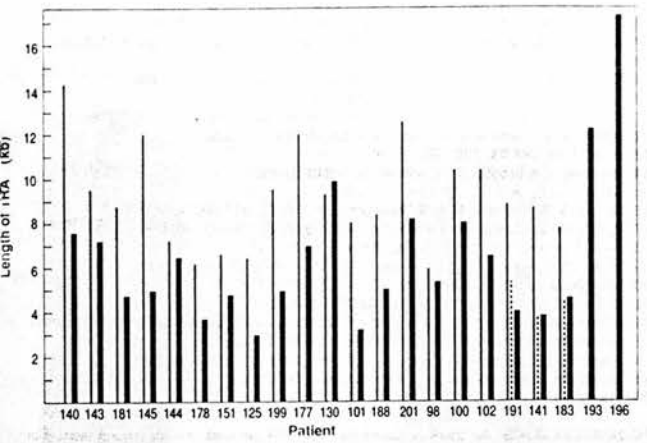


FIG. 1 Southern blot analysis of telomere repeat arrays in colorectal carcinomas and matched blood DNA samples. *a*, DNA was digested with the restriction enzyme *Hin*fI and hybridized with ^{32}P-labelled $(TTAGGG)_4$ probe. *b*, DNA was digested with the restriction endonuclease *Alu*I and hybridized with a ^{32}p-labelled $(TTAGGG)_4$ probe (left panel) or a $(CAC)_5$ probe (right panel). The filter was first hybridized to $(CAC)_5$ then stripped and rehybridized to $(TTAGGG)_4$. NM, normal mucosa; T, tumour; numbers refer to patient numbers. Size markers are indicated to the right (kb).
METHODS. Either *Hin*fI- or *Alu*I-digested total genomic human DNA (5 μg) was loaded onto a 0.8% agarose gel. After electrophoresis the gel was blotted to Hybond and hybridized to the radiolabelled probes. Hybridization was at 48 °C in 5 × SSC buffer and washes were at 48 °C in 4 × SSC.



IG. 2 Mean size estimates for the TRA in colorectal carcinoma, matched ormal mucosae and adenomas. —, Normal mucosa; ▬, carcinoma; - - -, denoma.

ETHODS. Autoradiographs such as those shown in Fig. 1 were analysed y an automatic autoradiographic scanner as described previously[7]. In all ases *Hin*fI-digested DNAs were analysed but similar results were obtained ith *Alu*I. The optical density reading under the peak was integrated and he mean determined. Usually a gaussian distribution was obtained and the ean position was very close to that at the centre of the peak. In all cases he values obtained for several different experiments were within 5–10% f each other. To avoid problems of over exposure of the signal, various xposure times were analysed. In some cases two separate peaks were bserved in the tumour samples (for example, 145), the larger peak, of ower optical density, similar in size to the corresponding normal mucosa. Ve have attributed this to contamination of the tumour by normal mucosa, or which there is independent evidence from allele loss studies. In these ases the mean size of the smaller peak is given.

Fig. 1*b*). DNA loading and integrity were checked by prehybridizing all blots to a $(CAC)_5$ minisatellite probe (Fig. 1*b*, right anel).

To provide an estimate of the degree of reduction of the TRA n tumours, densitometric scans were carried out on these utoradiographs (Fig. 1) and those obtained for 13 other umours and matched normal mucosae. The average TRA length n the 20 normal mucosae and matching carcinomas is depicted n Fig. 2; in all but one tumour (130) there was a reduction in he average TRA length relative to the normal mucosa.

In three patients we were able to study the length of the TRA n adenomas, which are the benign precursors of carcinomas. n all three cases (191, 141, 183) there was a reduction in the denoma similar in extent to that found in the corresponding arcinoma. In two carcinomas (193, 196) out of 35 studied, the elomeres were very much larger. Unfortunately, no matched ormal mucosae were available for these samples so we could ot determine if this represented an increase in telomere length n the tumour.

In several cases, the average TRA length is close to 3 kb, with nolecules ranging to 1 kb or less. After correcting for DNA oading using internal controls, there was a reduction in signal ntensity of about 90%, 90%, 80%, 65%, 65% and 20% for umours 181, 145, 178, 151, 143 and 144, respectively. Thus it reasonable to suppose that in a proportion of cells in tumours nd adenomas (Fig. 2) there will be one or several chromosome nds lacking G-rich repeats.

In a preliminary series of experiments we have observed TRA eduction in ovarian and lung carcinomas as well as the colon arcinomas reported here. Telomere shortening has also been eported in two Wilms' tumours[10].

What is the mechanism underlying this TRA reduction? Reduction could reflect the number of cell divisions it has taken o produce the tumour cells in the absence of telomerase function[2–4], which may be restricted to germ cells. We have compared the average TRA length in sperm with that in tissues from a 26-week human fetus and colon mucosa (Fig. 3) and blood (data not shown) samples analysed in this study. Although tissue from only a single fetus was studied, it is clear that TRA length in these tissues is close (1–2-kb shorter) to that found in sperm and considerably larger than that found in any colon mucosa (on average 12 kb shorter than sperm) or blood (on average 14 kb shorter) sample.

Firm figures are not available, but it is likely that the tissues of a developed fetus result from 20–50 cell divisions, whereas several hundred or thousands of divisions have produced the colonic mucosa and blood cells of 60-year old individuals[12]. Thus the degree of telomere reduction is more or less proportional to the number of cell divisions. It has been shown that the ends of *Drosophila* chromosomes without normal telomeres reduce in size by ~4 base pairs (bp) per cell division[13] and that the ends of yeast chromosomes reduce by a similar degree in a mutant presumed to lack telomerase function[14]. If we assume the same rate of reduction is occurring during somatic division in human tissues, then a reduction in TRA by 14 kb would mean that 3,500 ancestral cell divisions lead to the production of cells in the blood of a 60-year old individual; using estimates of sperm telomere length found elsewhere[10] we obtain a value of 1,000–2,000. These values compare favourably with those postulated for mouse blood cells[12]. Thus, we propose that telomerase is indeed lacking in somatic tissues. In this regard it is of interest to note that in maize, broken chromosomes are only healed in sporophytic (zygotic) tissues and not in endosperm (terminally differentiated)[15], suggesting that telomerase activity is lacking in the differentiated tissues.

Consistent with this idea, there is a significant reduction of TRA length as a function of age in human blood DNA, although there is a high degree of scatter (Fig. 4). From the regression analysis we can calculate that the rate of reduction is 33 bp per year. Extending this idea to colon tumours, a reduction in 5 kb would mean that it has taken ~1,000 cell divisions to produce a tumour from normal mucosa. Analysis of three cases (Fig. 2; 191, 141, 183) suggests that most of these doublings would take place in the formation of the adenoma. In tumours 193 and 196 we assume that the telomerase has been reactivated as proposed

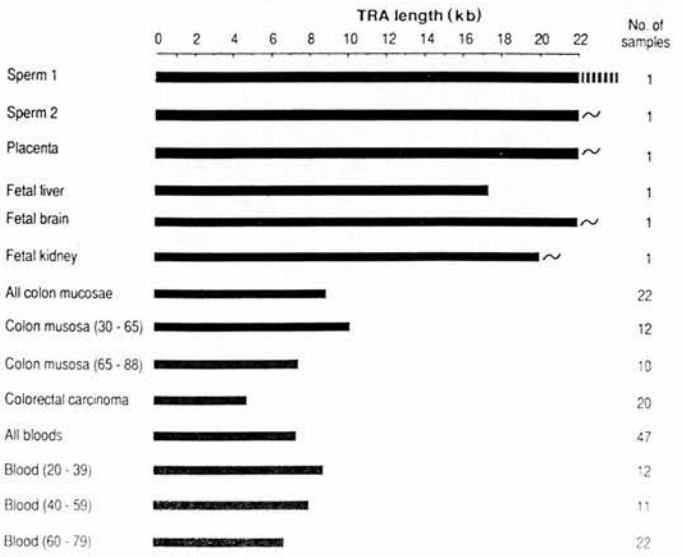


FIG. 3 Telomere length in different tissues and in association with ageing. The estimates are averages for each tissue where more than one sample was analysed. The data was obtained as described in Figs 1 and 2. Vertical bars indicate greater than, and the symbol ~ means approximately (values of ~20 kb and greater are approximations). Numbers in brackets in the left column refer to age range.

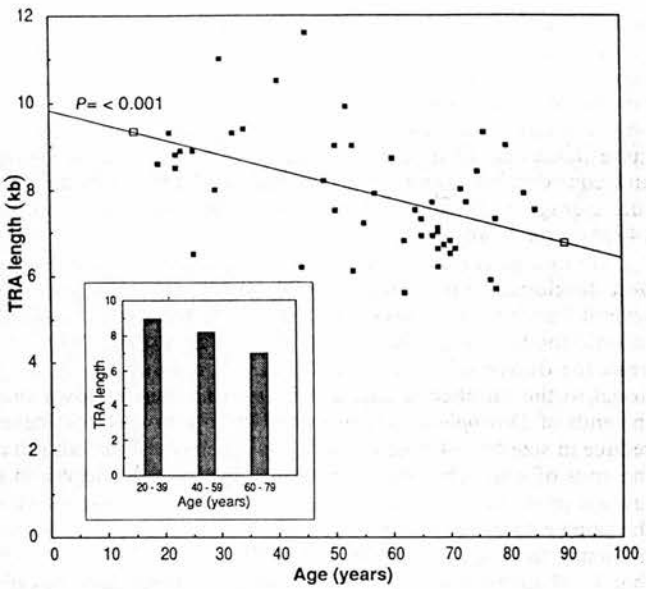


FIG. 4 Telomere reduction with age in human blood. The straight line was obtained by regression analysis. The significance of the regression, $t = -3.63$; $P < 0.001$. The slope is equivalent to 33 bp per year.

For HeLa cells in culture, which are known to contain telomerase activity[4,10].

One alternative explanation for our observations is that in tumours the cells with shorter telomeres have a growth advantage over those with larger telomeres, a situation described for vegetative cells of tetrahymena[16].

Telomere losses could be a consequence of tumorigenesis or be involved in causing malignancy. We believe there are two ways by which TRA losses could contribute to genetic instability, which is important for tumorigenesis. First, chromosomes lacking terminal repeats may be less stable and lost at higher frequency, a situation for which there is a precedent in the yeast EST1 mutant[14]. Second, chromosomes without telomeres may be prone to fusion-bridge-breakage cycles as described by McClintock[17], leading to daughter cells which receive partly deleted and partly duplicated chromosomes, respectively. There is no direct evidence to support this idea in carcinogenesis, although in senescent cells where chromosome fusions are found at high frequency[18] a loss of telomere repeats has been reported[19]. Also, telomere fusions occur in a range of tumours, including histiocytomas[20], cardiac myxomas[21], renal tumours[22] and leukaemias[23], as well as the common cancers at much lower frequency. In addition, broken ends within amplified dihydrofolate reductase genes lacking telomeres will fuse to other chromosomes, setting up breakage-fusion-bridge cycles[24].

Deletions or losses by these routes could play a part in generating the loss of alleles of restriction fragment length polymorphisms that often occurs in colorectal carcinomas and that is thought to reflect the need to render mutations in tumour suppressor genes homozygous[25,26]. Although some of these allele losses occur in the adenoma stage the majority are not apparent until the carcinoma stage[25]. In the three cases studied here (191, 141, 183; Fig. 2) we find that telomere reduction has already taken place by the adenoma stage. Hence these losses could be involved in causing the chromosome rearrangements subsequently observed in the carcinoma. □

Received 12 April; accepted 14 June 1990.

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ACKNOWLEDGEMENTS. We thank H. Cooke for fetal DNA samples and for helpful discussions, C. Potten and J. Ansell for discussions about stem-cell turnover, A. Carothers for statistical analysis, C. Harley, B. Futcher and C. Greider for communicating their work before publication, Professor H. J. Evans and Dr M. Steel and their support and discussions, D. Stewart and N. Davidson for photography and graphics and L. Campbell and E. Kay for secretarial assistance.

A truncated human chromosome 16 associated with α thalassaemia is stabilized by addition of telomeric repeat (TTAGGG)$_n$

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THE instability of chromosomes with breaks induced by X-irradiation led to the proposal that the natural ends of chromosomes are capped by a specialized structure, the telomere[1]. Telomeres prevent end-to-end fusions and exonucleolytic degradation, enable the end of the linear DNA molecule to replicate, and function in cell division (reviewed in ref. 2). Human telomeric DNA comprises ~2–20 kilobases (kb) of the tandemly repeated sequence (TTAGGG)$_n$ oriented 5′→3′ towards the end of the chromosome[3,4], interspersed with variant repeats in the proximal region[5]. Immediately subtelomeric lie families of unrelated repeat motifs (telomere-associated sequences) whose function, if any, is unknown[6,7]. In lower eukaryotes the formation and maintenance of telomeres may be mediated enzymatically (by telomerase)[8] or by recombination[9]; in man the mechanisms are poorly understood, although telomerase has been identified in HeLa cells[4]. Here we describe an α thalassaemia[10] mutation associated with terminal truncation of the short arm of chromosome 16 (within band 16p13.3) to a site 50 kb distal to the α globin genes, and show that (TTAGGG)$_n$ has been added directly to the site of the break. The mutation is stably inherited, proving that telomeric DNA alone is sufficient to stabilize the broken chromosome end. This mechanism may occur in any genetic disease associated with chromosome truncation.

During a search for novel α thalassaemia determinants, we identified a British man (II-1) with haemoglobin H (HbH) disease (HbH level 5.3%). This relatively severe form of α thalassaemia is usually caused by deletion of three of the normal

Am. J. Hum. Genet. 47:982–987, 1990

Genetic Linkage Map of Six Polymorphic DNA Markers around the Gene for Familial Adenomatous Polyposis on Chromosome 5

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Summary

A genetic linkage map of six polymorphic DNA markers close to the gene (*APC*) for familial adenomatous polyposis (FAP) on chromosome 5q is reported. One hundred fifty-five typed members of nine FAP kindred provided more than 90 meioses for linkage analysis. A number of crucial recombination events have been identified which are informative at three or more loci, allowing confident ordering of parts of the map. There was no evidence of genetic heterogeneity, with all families showing linkage of at least one chromosome 5 marker to the gene. Recombination data and two-point linkage analysis support a locus order of centromere–πD5S227–C11P11–ECB27–L5.62–*APC*–EF5.44–YN5.48–telomere, although EF5.44 could lie in the interval L5.62–*APC* or ECB27–L5.62. No recombinants were identified between *APC* and either EF5.44 or YN5.48, but published deletion mapping in colorectal carcinomas and linkage analysis in FAP suggest that YN5.48 is 1–3 cM from *APC*. The present study suggests that YN5.48 and L5.62 delineate a small region of chromosome 5 within which the EF5.44 locus lies very close to the *APC* gene. These data not only allow use of flanking markers for presymptomatic diagnosis of FAP but also provide a high-density map of the region for isolation of the *APC* gene itself and for further assessment of the role of chromosome 5 deletions in the biology of sporadic colorectal cancer.

Introduction

The genetic aberration resulting in familial adenomatous polyposis (FAP) has an incidence of approximately 1:10,000 (Reed and Neel 1955) but is of great clinical importance as a gene defect causing an autosomal dominant heritable cancer syndrome in which large-bowel cancer is preventable by prophylactic colectomy (Bulow 1987). The gene for FAP has been shown to be linked to markers which map to chromosome 5q (Bodmer et al. 1987; Leppert et al. 1987; Meera-Khan et al. 1988; Nakamura et al. 1988; Dunlop et al. 1989; Murday et al. 1989; Varesco et al. 1989) and is known as *APC*. *APC* has also been implicated as a tumor suppressor gene involved in the biology, if not in the genesis, of sporadic colorectal cancer. Loss of genetic material from chromosome 5 has been demonstrated in FAP and in sporadic colorectal adenomas and carcinomas (Solomon et al. 1987; Vogelstein et al. 1988; Ashton-Rickardt et al. 1989; Sasaki et al. 1989). The mechanism of such loss is usually by interstitial deletion (Ashton-Rickardt et al. 1989). The published data broadly support the hypothesis that *APC* mutations are recessive at the cellular level and, in common with other tumor suppressor genes, require inactivation of both alleles if malignancy is to supervene (Knudson 1971).

In addition to causing the full FAP syndrome, *APC* mutations may also be involved in the inheritance of susceptibility to non-FAB colorectal cancer. Familial clustering of colorectal cancer has been well described (Woolf 1958; Macklin 1960; Lovett 1976; Duncan and Kyle 1982; Lynch et al. 1985; Bonelli et al. 1988). Dominant inheritance of colorectal adenomas and of carcinomas may even account for the majority of cases of these neoplasms with a gene frequency in the general population as high as 19% (Cannon-Albright et al.

Received May 15, 1990; revision received July 25, 1990.

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0002-9297/90/4706-0015$02.00

1988). Clinically, certain extracolonic features of FAP occur with excess frequency in apparently sporadic colorectal cancer (Sondergaard et al. 1985*b*) and in hereditary nonpolyposis colorectal cancer (Sondergaard et al. 1985*a*), suggesting a common genetic etiology. In addition, genetic linkage has recently been established using chromosome 5q DNA probes in a family in which affected individuals have variable numbers of polyps which do not all meet the criteria for the diagnosis of FAP (Leppert et al. 1990). It seems likely that a number of different mutant genes are responsible for colorectal cancer susceptibility, and so *APC* may be considered a candidate member of such a group of genes.

A high density of polymorphic DNA markers in the region around *APC* will greatly facilitate efforts toward identification of *APC*. Even after full characterization of *APC*, it is likely that closely linked markers will still be valuable for confirmation of mutation analysis and for presymptomatic diagnosis of FAP in families with rare mutations.

The present study reports a genetic linkage map of six polymorphic DNA markers. Two new probes have been mapped, and one of these appears to be very close to *APC* itself.

Material and Methods

Nine Scottish kindreds with FAP were studied, comprising 155 genotyped and phenotyped individuals. This provided in excess of 90 meioses for linkage analysis if all families were fully informative for any marker. Clinical, pathological, and genealogical data were complete, and affection status and penetrance were assigned in the manner which we have described elsewhere (Dunlop et al. 1989).

Genotyping was carried out as we have reported elsewhere (Dunlop et al. 1989), and all techniques were carried out by established protocols. In brief, DNA was purified from fresh peripheral blood leukocytes, digested with the appropriate restriction endonuclease, agarose-gel fractionated, and transferred onto nylon membranes, and hybridization of ^{32}P-labeled DNA probe was revealed by autoradiography.

Genotypes were obtained for six DNA probes which recognize RFLPs on chromosome 5. Probe DNA fragments used were as follows: π227 (D5S37) (Stewart et al. 1987) as a 900-bp, *Hin*dIII/*Eco*RI fragment in πAN7; C11P11 (D5S71) (Bodmer et al. 1987) as a 3.6-kb, *Eco*RI fragment in pUC8; ECB27 (D5S98) (Murday et al. 1989; Varesco et al. 1989) as a 2.8-kb, *Sal*I fragment in phage lambda; pL5.62 as a 10.5-kb, *Bgl*II fragment cloned into the *Bam*HI site of pUC18; pEF5.44 as a 2.3-kb, *Msp*I fragment cloned into the *Acc*I site of pUC18; and YN5.48 (D5S81) (Nakamura et al. 1988) as a 2.4-kb, *Taq*I fragment cloned into the *Acc*I site of pUC18. Table 1 shows polymorphisms recognized by each of these probes and the appropriate restriction enzyme used in the present study. Allele sizes and frequencies from a local control population are also tabulated in table 1. Probes pL5.62 and pEF5.44 are DNA fragments recognizing polymorphisms as noted above and are subclones from cosmids selected from a genomic cosmid library derived from part of chromosome 5q (Nakamura et al. 1988).

Linkage analysis was carried out using the *LINKAGE* group of programs. The 1-lod unit of support was

Table 1

Allele Size and Frequency Recognized at Six Polymorphic Chromosome 5q DNA Marker Loci in a Control Scottish Population

Probe (enzyme recognizing polymoprhism)	Frequency/Size (kb) A1	A2	A3	No. of Chromosomes Examined
EF5.44 (*Msp*I)	.18/2.9	.82/2.1		84
L5.62 (*Bgl*II)	.93/9.0	.07/5.5		76
YN5.48 (*Msp*I)	.45/9.0	.55/8.0		60
ECB27 (*Bgl*II)	.39/11.9	.61/10.5		74
C11P11 (*Taq*I)	.14/4.4	.86/3.9		100
π227 (*Pst*I)	.25/3.0	.75/4.3		66
π227 (*Bcl*I)	.17/3.0	.46/1.8	.37/1.2	54
π227 (*Bst*XI)	.29/2.7	.71/2.3		28
π227 (*Mbo*I)	.25/.55	.75/.45		20

taken as representative of 95% probability limits. Multipoint linkage analysis was not felt to be valid for the present study, since only affected families were analyzed, without Centre Etude de Polymorphisme Humaine (CEPH) data. Instead, the data are presented as a series of pairwise analyses between marker and disease loci and between all pairs of marker loci. Crucial rare recombinant events informative at three or more loci were identified and have allowed confident locus ordering for some parts of the map. The lod scores for linkage between markers are relatively low because the families were not specifically selected for a high level of informativity such as is the case with CEPH families.

Validation of paternity and of typing for chromosome 5 markers was inherent in the multiple two-point analyses which were carried out. In addition, paternity testing was carried out on all *APC*-marker obligate recombinants by using the probe 29C1 (DXYS14), which is derived from the sex chromosomes and recognizes a pseudoautosomal hypervariable polymorphism (Cooke et al. 1985). This probe is extremely useful for paternity testing since typing can be carried out simply by Southern blot analysis. In addition, Cooke et al. (1985) found that, of 83 individuals studied, no two had the same genotype.

Results

The results of the analysis for linkage of *APC* to each of the six polymorphic DNA marker loci are presented in table 2. The peak lod score (Z_{max}) and recombination fraction (θ) indicating the maximum likelihood of marker location relative to *APC*, along with 95% probability limits, are tabulated together with lod-score tables. No recombinants have been identified between *APC* and YN5.48 or between *APC* and EF5.44. The Z_{max} for APC–YN5.48 was 7.00 at θ = .00, with 95% probability limits .00–.07. EF5.44 was not very informative in these families, and so the Z_{max} for *APC*–EF5.44 was only 3.5 at θ = .00, with resultant wide 95% probability limits.

Tests for linkage between each pair of marker loci revealed no discrepancies to suggest nonpaternity or mistyping. There were no cases of nonpaternity when all marker-*APC* recombinants were tested with the probe 29C1.

Lod-score calculations were carried out for linkage between each pair of markers, and these data are shown in table 3. The θ is given above the diagonal and the Z_{max} is given below the diagonal, for each marker-marker combination. No recombination events were detected between the following pairs of markers: L5.62–ECB27, ECB27–C11P11, C11P11–π227, and EF5.44–L5.62. However, the lod scores for EF5.44–L5.62 and for L5.62–ECB27 were low because of poor informativity of these markers in the local population (see table 1).

Table 4 shows all recombination events which were fully informative for three or more loci. The vertical line represents the recombination event in each case, and the informative markers are ranged either side of the breakpoint, depending on the haplotypes resulting from the recombination. Figure 1 shows this information in graphic form. The shaded area represents all possible *APC* locations, relative to the markers studied. Thus, *APC* could overlap EF5.44 or YN5.48 but cannot overlap L5.62, ECB27, C11P11, or π227, because of the presence of recombinants. These data suggest that YN5.48 and L5.62 delineate a small region which spans *APC*. The interval L5.62–YN5.48 could be as much as 11 cM (see table 3) or 7 cM (since L5.62–EF5.44 is 0 cM and since EF5.44–YN5.48 is 7 cM) or as little as 2 cM (since L5.62–*APC* is 2 cM and since *APC*–YN5.48 is 0 cM). The latter interval

Table 2

Linkage Analysis in Nine Scottish FAP Kindreds (KMD1–6, 8, 14, and 16)

Linkage vs. *APC*	Z_{max}	θ	95% Probability Limits	θ					
				.00	.05	.10	.15	.20	.25
EF5.44	3.50	.00	.000–.160	3.50	3.22	2.91	2.58	2.23	1.86
YN5.48	7.00	.00	.000–.070	7.00	6.31	5.56	4.79	4.01	3.24
L5.62	13.31	.02	.005–.095	. . .	12.99	11.94	10.65	9.21	7.65
ECB27	1.89	.06	.002–.260	. . .	1.88	1.76	1.50	1.20	.90
C11P11	5.45	.09	.025–.210	. . .	5.23	5.43	5.13	4.59	3.90
π227	4.40	.11	.035–.235	. . .	4.00	4.40	4.26	3.88	3.35

Table 3

Two-Point Z_{max} Values (below diagonal) and θ Values (above diagonal) for Linkage between Marker Loci in Nine FAP Kindreds

	EF5.44	YN5.48	L5.62	ECB27	C11P11	π227
EF5.44		.07	.00	.15	.16	.25
YN5.48	2.11		.11	.19	.11	.25
L5.62	1.23	.98		.00	.04	.04
ECB27	.69	.52	1.03		.00	.12
C11P11	.64	1.60	4.11	1.86		.00
π227	.00	1.05	1.48	1.72	3.05	

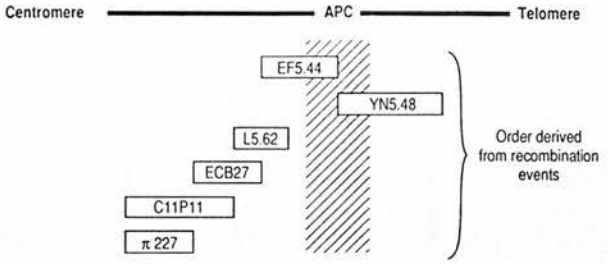

Figure 1 Markers ordered from recombination events as detailed in table 5. *APC* is represented by the shaded area, and the site of marker locus possible at any point in the box indicated.

(2 cM) seems most likely, since lod scores are highest for linkage of each of the markers to *APC*. When two-point linkage analysis is added to the order derived from recombination events (table 4), and given that C11P11 is centromeric to *APC* (Varesco et al. 1989) and that YN5.48 is telomeric to *APC* (Nakamura et al. 1988), these data support the locus order centromere–π227–C11P11–ECB27–L5.62–*APC*–EF5.44–YN5.48–telomere, although EF5.44 could lie in the interval L5.62–*APC* or, least likely, in the interval ECB27–L5.62.

Table 4

Recombination Events in Which Three or More Loci Were Informative

C11P11 π227	*APC* EF5.44 L5.62	C11P11	*APC* EF5.44 YN5.48
L5.62 π227	*APC*	π227	*APC* EF5.44 L5.62
ECB27 π227	*APC*	L5.62 C11P11 π227	YN5.48
ECB27 C11P11	YN5.48	ECB27	EF5.44 YN5.48
ECB27 π227	YN5.48	ECB27 C11P11 π227	EF5.44 YN5.48
EF5.44 ECB27 C11P11 π227	YN5.48	π227	EF5.44 YN5.48 ECB27

NOTE.—The vertical line represents where the recombination has occurred in relation to each of the informative loci.

Discussion

The present study has provided a genetic linkage map of six polymorphic DNA markers which lie very close to *APC* on chromosome 5q. The large number of meioses studied and the detection of a number of rare recombination events which were informative at multiple loci have allowed confident ordering of parts of the map. The possibility of double crossovers for these events is remote, since the genetic area under scrutiny is very small indeed. The localization of YN5.48 distal to *APC* (Nakamura et al. 1988) now seems certain, following deletional analysis in colorectal carcinomas (Ashton-Rickardt et al. 1989) and the recent identification of an *APC*–YN5.48 recombinant which was non-recombinant for APC–π227 (Tops et al. 1989). No *APC*–YN5.48 recombinants were detected in the present study, but, when the relevant two-point linkage analysis data are added into the map, the localization of YN5.48 distal to *APC* and on the other side from π227, C11P11, ECB27, and L5.62 is highly likely. The present study has not provided firm data on the exact location of EF5.44, but the latter certainly lies centromeric to YN5.48 and telomeric to ECB27. Because of this and the absence of *APC*–EF5.44 recombinants with a lod score of 3.50, we believe EF5.44 to be very close indeed to *APC*. In the search for relatively small deletions, use of EF5.44 to screen pulsed-field DNA fragments for rearrangements in patients with FAP could provide the key to isolation and characterization of *APC*.

The data presented here, combined with those already published for linkage of several of these markers to the *APC* gene, give lod scores sufficiently high (and hence 95% probability limits sufficiently narrow) to allow their use in presymptomatic diagnosis of FAP. Estimates of genetic distance from *APC* for the markers published as lod-score tables when data from this anal-

ysis are combined are as follows: YN5.48–*APC*, approximately 18.5 at $\theta = .025$ (Nakamura et al. 1988; Tops et al. 1989); π227–*APC*, 18.95 at $\theta = .075$ (Meera-Khan et al. 1988; Murday et al. 1989); and C11P11–*APC*, 11.97 at $\theta = .025$ (Bodmer et al. 1987; Leppert et al. 1987).

The establishment of a high-density map of the region around *APC* is of great importance, since, until isolation of the gene itself, multiple markers will be required for presymptomatic diagnosis of FAP. Even after *APC* is cloned and sequenced, it is likely that there will be a need for linked markers in some families. We have detected no evidence of genetic heterogeneity in nine Scottish FAP kindreds, with linkage being apparent for at least one of the six markers studied in all families; and, to date, there are no families in the world literature which do not exhibit linkage to 5q21–22. This is of paramount importance to clinicians who will use genetic linkage data to influence management of families and at-risk individuals under their care.

In the biology of colorectal carcinomas, the use of this high-resolution map will also be of great benefit for further examination of the role of loss of heterozygosity at loci close to *APC*. Examination of DNA from both FAP and non-FAP colorectal adenomas and carcinomas at the EF5.44 locus will also be of great interest, since the present study has demonstrated the close proximity of EP5.44 to *APC*. Preliminary data on such analysis have suggested that the frequency of EF5.44 loss is very high in colorectal cancer tissue (Wyllie et al. 1989). This again supports the linkage data presented here, suggesting that EF5.44 is very close to *APC*.

The present study has delineated between YN5.48 and L5.62 a small chromosome 5q region, which may be as little as 2 megabases. This region includes the gene for FAP, and we have identified a marker which we believe lies between the two flanking markers and appears to be very close to *APC* itself. It is now possible to proceed to isolate clones from this region and to screen these for the presence of expressed sequences. Any such sequences must be strong candidates for *APC* itself, given both the relatively small distance between these markers and that *APC* is probably a fairly large gene, since the rate of new mutations giving rise to sporadic cases of FAP is high (Reed and Neel 1955). Isolation of *APC* will only be the opening chapter of a fascinating book providing new understanding of a heritable cancer syndrome and also of the biology of colorectal cancer.

Acknowledgments

M.G.D. was funded by a Medical Research Council Training Fellowship. This work was partly funded by the Cancer Research Campaign. Thanks to the following for use of their probes: P. Scambler for C11P11, A.-M. Frischauf for ECB27, and G. D. Stewart for π227.

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Status of the APC Gene in Familial and Sporadic Colorectal Tumours as Determined by Closely Flanking Markers

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ABSTRACT

Using polymorphic close-flanking markers, allele deletion in the vicinity of APC was detected in the majority of sporadic colorectal cancers in a large unselected series. Deletion was due to interstitial deletion or mitotic recombination, but seldom to whole chromosome loss. Mitotic recombination affecting APC was associated with smaller tumours than interstitial deletion. Sporadic adenomas showed a slightly lesser frequency of allele loss, but no such loss was observed in 28 adenomas from 4 cases of familial polyposis.

INTRODUCTION

This paper presents new data on the incidence of deletion of the APC (Familial Polyposis) gene in human colorectal tumours. Accurate measurement of this incidence provides the only firm clues at present available to the functional significance of APC. In previous work, incidence of allelic deletion involving chromosome 5q is variously reported between 20% and 40% in sporadic colorectal carcinomas (Solomon et al 1987; Vogelstein et al 1988; Law et al 1989) in around 30% sporadic adenomas (Vogelstein et al 1988; Rees et al 1989), and in few if any adenomas of patients suffering from polyposis coli. Only one group has presented convincing data on the status of 5q alleles in carcinomas from polyposis patients, allele loss being observed in around 24% (Okamoto et al 1988; Sasaki et al 1989). In all of these studies, however, the analysis of 5q alleles was based on relatively few, dispersed loci. In this paper we describe a large series of sporadic carcinomas, and substantial numbers of sporadic and familial adenomas, using a group of probes detecting polymorphic sequences tightly clustered around APC.

A CLUSTER OF PROBES LINKED TO APC

Maximum lod scores and the corresponding recombination fractions are shown for five probes tightly linked to APC as tested in nine Scottish polyposis kindreds involving more than 90 meioses (Table 1). Critical recombinations indicated that π227 and C11P11 are on the same side of APC,(Dunlop et al 1989), whereas YN5.48 is on the opposite side. Physical maps suggest that the probes lie in the order: cen-π227-C11P11-L5.62-EF5.44-YN5.44-MC5.61-ter.

LOSS OF APC ALLELES IN SPORADIC COLORECTAL CARCINOMA

π227, YN5.48 and MC5.61 detect highly polymorphic sequences, so that the majority of patients studied are informative. EF5.44 and L5.62 are less informative. Accordingly, we initially studied a series of 146 sporadic colorectal carcinomas with π227, C11P11, YN5.48 and MC5.61 to determine the frequency of allele loss close to APC

TABLE 1

APC LINKAGE DATA DERIVED FROM STUDY OF 9 SCOTTISH POLYPOSIS KINDREDS.

probe tested with APC	max. lod score (Z)	recombination at Z (θ)	95% probability limits (θ)
π227	4.40	0.11	(0.035-0.235)
C11P11	5.45	0.09	(0.025-0.21)
L5.62	13.31	0.02	(0.005-0.095)
EF5.44	3.50	0.00	(0.00-0.16)
YN5.48	7.00	0.00	(0.00-0.07)

(Ashton-Rickardt et al 1989). 53% of the 85 tumours informative on both sides of APC showed loss of the centromeric or telomeric flanking markers, or both. We then focussed on those tumours in which the first four polymorphic flanking markers had failed to detect allelic losses. Of the 40 tumours in this category, 22 were informative at the L5.62 or EF5.44 loci, and 5 showed allele loss. Hence the true overall frequency of APC-linked allele loss detected by these probes is 53% + {47% x 5/22}, i.e. 63%. This frequency can only rise further as probes closer to APC are employed. Hence APC joins the putative anti-oncogene loci on 17p and 18q (Fearon et al 1987; Vogelstein et al 1988; Monpezat et al 1988; Lothe et al 1988) in showing allele loss in the majority (and perhaps being abnormal in all) of sporadic colorectal carcinomas.

THE CHROMOSOMAL LESIONS RESPONSIBLE FOR APC LOSS

Use of the probes listed above, together with probes hybridising to more distant loci on both long and short arms of chromosome 5 permitted description of the chromosomal lesions most commonly involved in APC allele loss. There were three major findings.

Firstly, whole chromosome loss is rare. Hence 5q allele loss in colorectal cancers can seldom be the result of chromosomal nondisjunction, although this accounts for much of the aneuploidy of such tumours, as judged from the reported karyotypes (Reichmann et al 1981). Aneuploidy appears in the course of tumour progression, being rare in adenomas, but present in at least 60% of carcinomas (Goh & Jass, 1986; Quirke et al 1987). APC loss therefore appears to precede this stage of tumour progression, and this is confirmed by the observation that 5q allele loss was found in equal frequency in DNA-diploid and DNA-aneuploid carcinomas (Ashton-Rickardt et al 1989). Anecdotal evidence from other groups confirms this view (Remvikos et al 1988).

Secondly, the common causes of APC-linked allele loss are interstitial deletion and mitotic recombination (or conceivably partial chromosomal arm loss, which is not distinguished by this method of analysis). Many interstitial deletions are relatively small. Amongst the tumours in which APC-linked alleles are lost by interstitial deletions, the site most commonly involved (in informative tumours) is that detected by EF5.44. This completely accords with the suggestion from linkage and physical mapping data that this genomic sequence lies close to APC.

irdly, and quite unexpectedly, tumours in which APC alleles are
through mitotic recombination are significantly smaller than
urs in which the allele loss results from interstitial deletion.
difference persists after excluding from the analysis all
t-sided colonic carcinomas, which may tend to present later than
e in the rectosigmoid. This finding underlines the importance
he status of APC in colorectal tumour growth, and may result
a gene dosage effect.

ALLELE LOSS IN ADENOMAS

series of 63 sporadic adenomas from 40 patients informative at
site on 5q, we identified 19 (30.2%) with allelic deletion. The
ysis of these adenomas has not involved as many loci as in the
inomas, but comparison of the incidence of allele loss at three
morphic loci confirmed the trend that adenomas show less allelic
tion than carcinomas. Differences in incidence of allele loss
een carcinomas and adenomas were significant when tumours with
rmative flanking markers on both sides of APC are compared. In
rast, none of 28 adenomas from four patients with polyposis
ed allele loss. Although many of these were smaller than the
adic adenomas, several showed features normally regarded as
ifying aggressive behaviour: aneuploidy and severely dysplastic
ology.

LUSIONS

data support the view that allele loss at the APC locus is a
ly significant and common event in human colorectal
inogenesis. Single allele abnormality (as in polyposis adenomas)
ars to be adequate to permit development of adenomas, presumably
onjunction with some additional event, acquired during exposure
arcinogens in the gut lumen. The majority of carcinomas have
nstrable allele loss (and presumably point mutations in the
dual allele) at APC. Similar abnormalities frequently occur in
adic adenomas, and may permit their progression to carcinoma.
e findings, and also the observation that major tumour
ploidy develops after 5q allele loss in many sporadic
inomas, show that APC abnormalities affect colorectal mucosal
helium at an early stage in carcinogenesis. The mechanism
eby the alleles are lost, however, appears to influence the
equent growth pattern of the tumour.

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JOURNAL OF PATHOLOGY, VOL. 162: 000–000 (1990)

REVIEW ARTICLE—CHROMOSOME PATHOLOGY

ALLELE LOSSES AND ONCO-SUPPRESSOR GENES

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INTRODUCTION

Our present knowledge suggests that aberrations f two major classes of genes are involved in the enesis and progression of malignancy. Abnormal ctivation and/or aberrant expression of the gene roduct characterize the first class of altered genes hich act dominantly in producing the malignant henotype. These are known as oncogenes. Loss of ene function is the feature which implicates the econd and perhaps more heterogeneous group of enes in carcinogenesis. These genes have been nown to have onco-suppressor activity and have een labelled anti-oncogenes but the preferred term tumour suppressor genes. Both copies of such enes require to be inactivated and so the causal nutations are recessive at a cellular level. Tumour uppressor genes are involved in regulation of cell roliferation or differentiation but the mechanism f such regulation remains speculative for the pres-nt. It may be that tumour suppressor genes have ome cellular stabilizing activity, indirectly balanc-ng the biological driving forces of oncogenes. This eview will deal only with the scientific background nd the detection of tumour suppressor genes, and ot with the complex question of what such genes ight do.

The first evidence for tumour suppressing genes ame from the cell fusion experiments of Harris and Klein.[1] Fusion of normal mouse fibroblasts with tumorigenic cell line resulted in extinction of umorigenicity in the resultant hybrids so long as pecific chromosomes were not lost. The expulsion f certain chromosomes (especially mouse chromo-ome 4, homologous to human chromosome 1) esulted in the reappearance of the tumorigenic henotype. Subsequent studies with human intra-pecies hybrids have suggested tumour suppressor ctivity on chromosomes 1, 2, 4, 11, 13, ,17, and 20.[2] It is interesting to note that the earliest experiments involving such cell hybridization techniques predate the discovery of oncogenes by 20 years!

A great deal of normal genetic material is transferred by cell fusion techniques and so the specific involvement of any particular onco-suppressor locus is difficult to assess. This problem was partially circumvented by the introduction of a single copy of chromosome 11 into a tumorigenic HeLa-human fibroblast hybrid (which lacked chromosome 11). This resulted in suppression of tumorigenicity in the resultant hybrid.[2] More recently, transfection of the cloned retinoblastoma gene into a tumorigenic retinoblastoma cell line using a retroviral vector has been shown to suppress the malignant phenotype.[3] If this finding is borne out by further studies, this is the first direct evidence that the retinoblastoma gene does indeed have tumour suppressing activity.

It seems likely that inactivation of specific tumour suppressor genes may be involved in the genesis of particular tumours or in individual steps towards the malignant phenotype in a particular class of tumours. However, deletion of certain genes may well be a common molecular end point in the biology of progression and metastasis of many tumour types.

Cell fusion models of tumorigenicity strongly support the existence of onco-suppressor genes but there is also a growing body of corroborrating evidence *in vivo*. Knudson's statistical analysis of the epidemiology of retinoblastoma[4] led him to suggest that only two mutations were required for both the heritable and the sporadic forms of the disease, the first mutation being germline in the inherited disease and affecting the somatic cell in the sporadic form. The second mutation would invariably be somatic and 'unmask' the mutant allele on the other chromosome. Thus, although the gene defect may be inherited in a dominant mode, its action is

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cessive at a cellular level since both copies require be inactivated. This hypothesis has subsequently een confirmed by molecular analysis. Retinoastoma tumour tissue has been shown to have omozygous mutations in the Rb gene and individls with the heritable form of the disease have herited a constitutional heterozygous Rb gene utation.[5] Retinoblastoma and another childhood ncer, Wilms' tumour, now serve as paradigms for l such heritable cancer syndromes and their adult ounterparts. These include familial adenomatous olyposis/colorectal cancer and hereditary/sporadic east cancer.

Epidemiological support for a two-hit, or recessve, theory of oncogenesis has been evident for any years. Armitage and Doll are widely quoted having demonstrated the involvement of several utational steps in carcinogenesis when they sumed a variable level of environmental carnogen. However, they re-examined their data, suming a constant exposure to carcinogen, and blished an analysis favouring a two-mutation pothesis in 1957.[6] This model accounts for the served incidence of many of the common adult ncers, notably breast and colon cancer.

Two mutations involving both alleles of recessive mour suppressor genes are clearly not sufficient to oduce all of the molecular changes described in mmon adult malignancies such as colon cancer.[7] owever, a two-step theory of mutagenesis would for the critical rate-limiting steps along the pathay to selection for a malignant clone. Events at her loci may confer further growth advantage and t as tumour promoters. It is likely that the initiatg mutation renders the proliferating clone more sceptible to such subsequent mutations. Several mour suppressor genes may require homozygous activating mutations and several oncogenes reuire abnormal activation before the establishent of a clone capable of all of the clinical anifestations of malignancy.

Homozygous deletion of a putative tumour ppressor gene does not invariably implicate that ne as conferring an inherited predisposition to at cancer. Indeed, the gene for the inherited rm of Wilms' tumour does not map to the region n chromosome 11 which is commonly deleted in mour tissue. However, regions of deletion in mour tissue and chromosomal locations of the rresponding inherited cancer predisposing gene ave been shown to coincide in many cases. In ncers where deletions of multiple loci have been oted, germline mutations of any of these putative tumour suppressor genes might confer predisposition to that malignancy. Therefore, familial predisposition to the common adult cancers may be very heterogeneous at a molecular level.

Inherited mutations tend to be small deletions or point mutations but these are extremely difficult to identify. The second event often involves loss of a more substantial piece of chromosome. It follows that demonstration of a consistent loss of genetic material from a particular chromosomal locus in tumour tissue may serve as a signpost to a mutated tumour suppressor gene on the remaining homologue. This indirect approach of searching for germline mutations by looking for allelic deletions in the corresponding tumour tissue has proven invaluable in tracking down genes for several heritable cancer syndromes including colon cancer (familial adenomatous polyposis), von Hippel–Lindau syndrome, and multiple endocrine neoplasia (MEN 1).

There are two main methods used in the search for the relatively large deletions seen in tumours which represent the second event at putative oncosuppressor loci. These are tumour cytogenetics and the demonstration of loss of cloned DNA markers at regionally mapped loci. Tumour karyology is technically arduous and suffers from inherent limitations. Only very substantial losses of genetic material are visible microscopically and homozygotization of a mutant allele by chromosomal reduplication is invisible to the cytogeneticist. Therefore, only allele loss of DNA markers as detected by the techniques of Southern blotting and DNA hybridization will be considered further.

METHODS

The basic techniques of molecular biology are now well established and the commercial experimental systems are so refined that a complete novice can now expect high quality data after a short training period. Most biochemistry or microbiology laboratories will already have the basic equipment for Southern blot and DNA hybridization experiments. A gel electrophoresis tank, access to facilities for the use of radio-isotopes, and the use of a controlled room for cloning DNA probes in bacteria are the only requirements over and above what is normally available in such laboratories. For a manual of the practical procedures involved see Maniatis *et al.*[8] as an example.

DNA is purified from tumour tissue and from normal tissue representing the constitution of the

lividual to serve as a control. The constitutive
NA can either be from peripheral blood leuko-
es or from normal tissue adjacent to the tumour.
wever, there is the possibility that the mutation
y occur as a field change within the affected organ
d so it is probably best to include both forms of
ntrol. The purified DNA is digested with a restric-
n endonuclease and the resultant fragments are
e-fractionated by gel electrophoresis. The DNA
hen transferred to a nylon membrane by South-
n blotting and immobilized there by ultra-violet
ht. A cloned DNA fragment (which can be a tran-
ibed gene or an anonymous sequence) is labelled
th phosphorus-32 incorporated nucleotide,
ually cytidine. This radiolabelled DNA probe is
n hybridized to the fractionated total genomic
NA on the filter. The DNA probe will hybridize to
complementary base sequence on the filter and
s is detected by autoradiography.

There are two ways to identify loss of genetic
terial at the chromosomal locus homologous to
DNA probe used in the hybridization. The first
these is by the technique of gene dosage. Each of
two chromosomal homologues carries one allele
the DNA probe sequence. Loss of one allele
be detected by demonstration of a 50 per cent
luction of signal intensity by laser densitometry
en comparing the hybridized band derived from
nour DNA with that of the control leukocyte
NA. A control probe homologous to a non-coding
quence or gene at an unrelated locus must be
d to compare signal intensities to ensure that
reduction is not due to unequal loading of the
ested denomic DNA samples. However, this
hnique is subject to certain limitations. In some
nours, the mechanism of homozygotization for
mutant allele has been shown to involve mitotic
combination and reduplication of the abnormal
ne. No reduction in band intensity would then be
parent, since two alleles would be equally rep-
ented even though both were associated with
ne mutations. This problem can be overcome by
amining only those loci which exhibit natural
lymorphisms. These polymorphisms reflect dif-
ences in the amount of DNA between adjacent
triction endonuclease cleavage sites. This vari-
on can be due to the presence or absence of a
rticular restriction site or to a variable amount of
n-coding DNA repeat sequences, known as vari-
le number tandem repeat (VNTR) or minisatellite
quences, between adjacent restriction sites. If any
dividual carries one allele of different length on
ch of the two chromosomes, then that individual
is constitutionally heterozygous at that locus. The two alleles migrate differentially on gel electrophoresis and are thus a representation of the paternally and maternally derived chromosomes. Any loss of genetic material at the test locus is apparent if one of the alleles is absent when a DNA probe homologous to the deleted locus is hybridized to the genomic tumour DNA digested by restriction endonuclease (Fig. 1). This is known as loss of constitutional heterozygosity and it is the allele associated with the *abnormal* gene that is retained. If both of the bands are absent, there is homozygous deletion of that region and this implies that the locus corresponding to the probe sequence is very close indeed to the target gene and may even lie within the gene itself. Reduplication of the abnormal chromosome will not affect the demonstration of loss of heterozygosity, since only the allele associated with the mutation will be copied and the tumour will still appear homozygous for that allele.

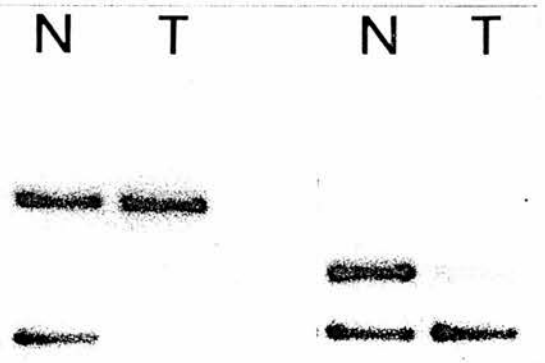

Fig. 1—Allele loss in tumour tissue. Total genomic digests of leukocyte DNA (N) and colonic carcinoma DNA (T) from two patients. The DNA was digested with *Hinf 1* restriction endonuclease, electrophoresed in agarose. A highly polymorphic radiolabelled chromosome 5 DNA probe, λMS8, was hybridized to the genomic digests after transfer to a nylon filter. In each case, there is loss of constitutional heterozygosity at the λMS8 locus in the tumour DNA, demonstrated by the absence of one of the alleles. In one of the tumours, there is a faint hybridization signal where the absent band should be, due to the presence of contaminating normal stroma and leukocytes in the tumour specimen

A low level of non-specific allele losses occur throughout the genome in the somatic tissues of many tumours and so identification of specific gene deletions requires the demonstration of consistent,

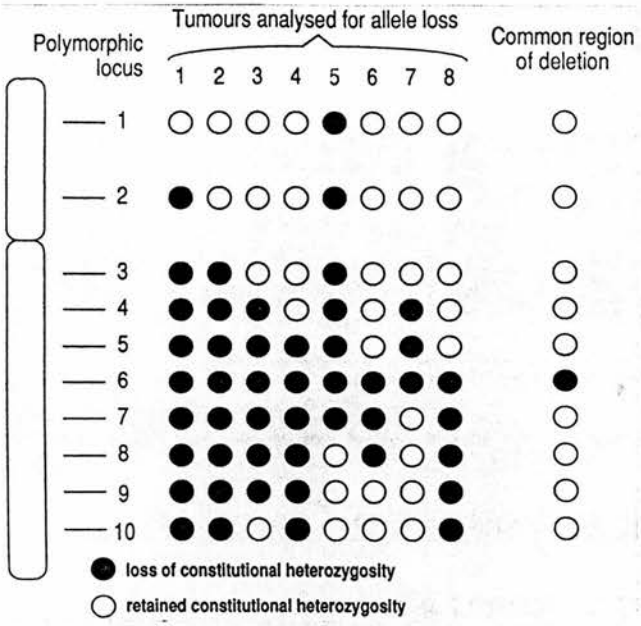

Fig. 2—Localization of a tumour suppressor gene by deletion mapping. The basic principle of construction of a composite deletion map is demonstrated. Multiple polymorphic loci are examined for allele loss in several tumours (in this case, eight tumours, though many more would be required in practice). A common region of deletional overlap will be identified which is the smallest portion of chromosome which must harbour the tumour suppressor gene of interest

on-random losses. This generally means the examıation of large numbers of tumours and the demontration of allele losses in excess of an empirical equency of around 10 per cent. Demonstration of on-random allele losses have been reported in reast cancer (chromosome 11p, 13q, 17p) colon ancer (5q, 17p, 18q), osteosarcoma (13q), and ıany other malignancies, all pointing to possible ıvolvement of tumour suppressor loci in those ımours.

Losses of quite substantial portions of chromoɔmes are quite frequently seen in individual tuıours and many genes are deleted in addition to ıe target gene. It is then difficult to invoke the ɔecific involvement of a particular gene within the eleted area as a non-functioning onco-suppressor. Iowever, by examining multiple polymorphic loci panning the region of interest and by using the llele loss pattern from a large number of tumours, composite deletion map can be constructed which ıay reveal a specific minimum region of common eletion (Fig. 2). This smallest region of deletional ıverlap can allow the localization of the putative umour suppressor gene to within the distance between the closest markers flanking the gene. However, this might be several millions of base pairs. For the present, deletion mapping is a relatively coarse tool when applied to the majority of the genomes and relies on the mapping of DNA probes by family studies using linkage analysis or by physical mapping techniques. As the number of mapped and cloned DNA sequences increases, the level of refinement of the technique will improve commensurately.

APPLICATIONS

A search for allele losses can have two aims: either to identify chromosomal loci which have potential tumour suppressor activity by the demonstration of non-random losses, or to localize known oncesuppressor loci more accurately using deletion mapping as detailed above. The practical value of such a strategy for identification and further localization of a recessive tumour suppressor gene has recently been elegantly demonstrated by Baker *et al.*[9] A high frequency of non-random allele losses of the short arm of chromosome 17 in colorectal carcinomas

.d previously been demonstrated by that group. ıis corroborated cytogenetic findings of consistent -sses of that chromosome.[10] Baker *et al.* then local-ɔd the smallest common region of deletion using polymorphic DNA markers and analysing these -r loss of constitutional heterozygosity in 58 colo-ɔtal carcinomas. The smallest region of deletion -ways involved the gene for p53. Mutant forms p53 had previously been implicated in cellular -transformation and immortalization, suggestive involvement in carcinogenesis. Therefore, the -maining allele of p53 required careful examin-ion to assess whether there was a mutation on that -tained gene copy. The base-pair sequence of p53 -as already known and so Baker sequenced the p53 ɔne by producing a cDNA clone from the mRNA ɔing produced by the remaining p53 allele in ɔo colorectal cancers. In both tumours, point utations in coding sequences in the gene were ɔtected. Previous work on p53 function had ıggested that mutant forms of the gene have a ɔminant, oncogenic activity. However, Baker has -plained her findings by proposing a competitive ıtagonism of the mutant p53 over the wild-type rm. The loss of the normal allele would then ıve a more marked effect. Clearly the distinction ɔtween oncogene and tumour suppressor gene has ɔw become somewhat blurred. Although there is ill no proof that the p53 protein produced by the utant allele is biologically inactive, this study ɔes lend support to a recessive role for tumour sup-ɔessor genes in oncogenesis although the mechan-m of action may be more complex than was at first ɔlieved. The study also demonstrates a practical ɔproach to understanding the molecular biology many of the common cancers.

Once allele losses have been recognized as consistent findings in a particular cancer, localization has been refined by deletion mapping, and any mutations of putative onco-suppressor genes have been characterized at a molecular and functional level, the next step is to assess whether the gene might be involved in the genetic predisposition to that cancer. This may prove challenging since predisposition to any particular cancer may well involve different loci in different families. However, if onco-suppressor genes are involved in the inheritance of an increased risk of cancer, such findings will have exciting and far-reaching implications for the screening, diagnosis, and treatment of cancer for future generations.

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A73698PAT 00 000019587 0456 00482

In press, Lancet Dec. 90

CLINICAL APPLICATION OF LINKED DNA MARKERS FOR PRESYMPTOMATIC DIAGNOSIS OF FAMILIAL ADENOMATOUS POLYPOSIS

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Summary

DNA analysis was applied to preclinical diagnosis of familial adenomatous polyposis (FAP). 41 at-risk individuals (aged 0-39 years) were genotyped for 6 linked DNA probes and probe-derived risk assigned using the LINKAGE program. Probes flanking the gene were informative in 28 individuals (68%; 95% CI, 53.8-82.8%). Retinal examinations for congenital hypertrophy of the retinal pigment epithelium (CHRPE) and colonic screening were performed. 15 individuals were affected on screening and 14 (93%) of these had been assigned a probe-derived risk of >0.93. High risk estimations were assigned to 4 individuals previously discharged from follow-up but who were screened affected during this study. Residual risk was calculated for those screened negative by combining genotype data, and colonic and CHRPE findings. Residual risk of <0.001-0.003 was assigned in 89% of cases and <0.0001 in 56% of cases. This study demonstrates the clinical value and validity of risk analysis using linked DNA probes combined with retinal examination for CHRPE and we make recommendations for integrated screening programs for FAP.

Introduction

The gene responsible for Familial Adenomatous Polyposis (APC) was first mapped to the long arm of chromosome 5 by demonstration of genetic linkage to the DNA probe, C11P11[1,2]. Subsequently a number of chromosome 5 markers recognising DNA polymorphisms have been shown to be linked to APC[3-5] and we have reported a high resolution linkage map which comprises loci recognised by 6 DNA probes located close to, and on both sides of, APC[6]. Identification of APC-linked DNA markers will ultimately lead to isolation and cloning of the gene itself. However, there is a more immediate clinical benefit from such markers since they can now be used for preclinical risk estimations of Familial Adenomatous Polyposis (FAP) to influence the management of at-risk individuals in affected families.

The offspring of patients affected by FAP have a 50% chance of inheriting the gene defect and are known as at-risk family members. Intensive screening by regular endoscopy of the large bowel in such individuals is recommended from around the time of puberty until the age of 30 years but screening should continue until 60 years of age[7,8]. This burden could be reduced by analysis of DNA purified from a single blood sample using linked DNA marker probes. This would provide a simple method of presymptomatic screening for FAP in suitable families and give an assessment of risk independent of clinical screening. There are substantial numbers of individuals who could benefit from such DNA analysis. If the birth rate in the United Kingdom is taken as approximately 700,000 per annum and the incidence rate of FAP as 1 in 8,000 live births, then there are around 2600 affected individuals currently under 30 years of age and a further 90 are born each year. However, since new *APC* mutations make up 30% of all cases and such individuals are not suitable for preclinical diagnosis using linked DNA markers, these figures have to be modified accordingly. In addition, FAP is an autosomal dominant heritable disorder where, overall, affected parents have roughly equivalent numbers of affected and unaffected offspring but all of whom require screening. Therefore, one can calculate that there are currently 3700 at-risk individuals in the United Kingdom who would benefit from DNA analysis by linked markers with a further 126 born annually.

There is an inherent inaccuracy in the use of *APC*-linked probes for diagnostic purposes. This inaccuracy is due to the occurrence of crossing-over of genetic material between homologous chromosomes at the time of meiosis and is a function of the genetic distance between the marker locus and the *APC* gene on chromosome 5. The proportion of such crossing-over between marker and *APC* out of all meioses studied is known as the recombination fraction. If there is a recombination fraction of 5% between a probe and *APC*, then the probe will predict disease status correctly in 95% of tests carried out. Hence linked markers give an assessment of risk, not an absolute diagnosis. The risk estimation can approach 100% when markers mapping to both sides of the APC gene are used[6] (flanking markers). To make a completely inaccurate risk assessment when flanking markers are informative, there would have to be simultaneous recombinations between both markers and *APC*. This is highly unlikely and the chance of such an occurrence can be calculated as the product of the recombination fractions between each marker and *APC*. Therefore in the presence of informative flanking markers which both have recombination fractions of 5% for linkage to APC, the diagnostic accuracy can be calculated as 99.75%. In addition, any recombination event occurring between one marker and *APC* would be revealed by the other marker and this would be included in the risk analysis.

Application of genotype data from *APC*-linked probes could allow preclinical risk estimation in FAP which may substantially reduce the frequency of screening procedures required for those with low risk. Those assigned a high risk

could be informed of the likelihood that they had inherited the mutant gene and followed up until polyps appeared when surgical intervention could be planned.

We report the first clinical evaluation of the application of linked DNA probes to presymptomatic diagnosis of FAP. The study demonstrates the clinical value and validity of risk estimations derived from genotype data combined with clinical risk assessment by colonic examination and retinal examination for Congenital Hypertrophy of the Retinal Pigment Epithelium (CHRPE) and we make recommendations for changes to current screening policy.

Methods

This study consists of a prospective assessment of the clinical value and suitability of DNA analysis for preclinical diagnosis of FAP. In brief, clinical risk derived from age at last negative colonoscopy was combined with risk derived from linked DNA markers by Bayesian methods[9] and a round of clinical screening then carried out to assess whether the combined risk assigned for each individual was indeed valid.

Forty one members of 7 families were identified as being at risk of FAP. Eleven (27%) of the study population had previously undergone examination of the colon and were thought to be unaffected. The risk that an individual is affected reduces with increasing age at each negative colonic examination. Valid estimation of such age-dependent risk can only be made from age-of-onset curves derived from a population of patients who have previously been screened negative, but subsequently develop polyps[10]. The liability classes delineating age-dependent penetrance used for this study are shown in Table 1 and are derived from the age-of-onset curves of Murday[10]. The prior risk for each individual screened is shown in Table 3 and was assigned as 0.5 for children or adults who had never been screened previously. Risk was assigned for those who had previously been examined as <0.5 depending on the age at last negative screen. For example the prior risk for an individual last screened negative at age 25 was 0.10, or a 10% chance of being affected.

Blood samples were then taken and genotypes obtained for each individual and all members of that family using 6 DNA probes for which we have recently reported a genetic linkage map around *APC*[6]. Genotyping for RFLP's was carried out by established protocols[11]. In brief, DNA was purified from peripheral blood leukocytes, digested with the appropriate restriction endonuclease, size-fractionated by agarose gel electrophoresis, transferred and immobilised on nylon membranes, and hybridised with radiolabelled DNA probe. The relative mobilities of fragments of genomic DNA complementary to the probe were then revealed by autoradiography at -70°C for 24-48 hours. Details of DNA probes used in this study are shown in Table 2. The order and recombination fractions for linkage of the marker to *APC* were derived from independent data including published linkage analyses[1-6,12], *in situ* hybridisation analysis[5] and deletion analysis in sporadic colorectal carcinomas[13]. Published data on locus order are now secure and that order is pi227-C11P11-ECB27-L5.62-APC-EF5.44-YN5.48. Estimates of recombination fractions between adjacent loci were based on our own mapping data[6] combined with published linkage analyses[1-5,12] and were taken as 0.03, 0.03, 0.02, 0.02, 0.01, 0.02 for the intervals in the order of loci given above. Combined published data on 95% probability limits for linkage of probes pi227, C11P11, L5.62 and YN5.48 to *APC* are narrow and so have not been taken into account in risk analysis. In addition, probability limits have little influence on risk estimation when close flanking markers are informative and when independent clinical and genetic risk estimations are combined, as reported here.

Risk estimations for genotype data were initially calculated by hand to ensure there were no genotype inconsistencies in tabulating and entering the data into the computer. Formal analysis was then carried out using multipoint linkage analysis with the MLINK program in the LINKAGE 5.02 group of programs on an IBM Series 80386 computer with math coprocessor. In one large family, computation times were up to 4 hours due to a number of unknown genotypes in early generations.

A preliminary risk estimation was calculated on computer by combining genotype data and initial clinical risk. A round of clinical screening was then undertaken to assess the validity of this initial risk estimation. All at-risk family members over 13 years of age were invited for screening of the large bowel by colonoscopy or by rigid sigmoidoscopy and barium enema. All polyps were biopsied for histological confirmation. In addition to colonic screening, affectation status was also assigned according to the presence or absence of congenital hypertrophy of the retinal pigment epithelium (CHRPE)[15,16]. Individuals with multiple, large and/or bilateral lesions were considered to be gene carriers while those with less than 3 lesions were considered negative as suggested by Chapman[16]. Retinae were assessed by two independent observers by direct and indirect ophthalmoscopy. All CHRPE lesions were photographed using a fundus camera and there were no inconsistencies in assigning presence or absence of the CHRPE phenotype.

Empirical assignment of a penetrance value for CHRPE was required as there are conflicting published data. One study has suggested that expression of CHRPE tends to co-segregate faithfully with colonic manifestations of the gene defect[16]. However, other reports suggest around 90% of affected individuals in any given family exhibit CHRPE as part of the FAP phenotype[17-19]. Therefore, penetrance has been taken as 0.90 in order to minimise any possibility of false reassurance to patients. Family members were also assessed clinically and radiologically for the presence of cranio-facial osteomas. Orthopantomography was not carried out in children. Two children were clinically assigned as gene carriers on the basis of gross cranial osteomas alone.

Clinical screening was completed in 34 individuals (83%) using any modality; 18 (43%) by colonic examination, 24 (59%) by ophthalmoscopy and 9 (21%) by radiological assessment for cranio-facial osteomas (see Table 3). Clearly the final clinical diagnosis based on colonic, retinal and bony findings for those who are affected is a hard end-point against which to evaluate the genotype-derived risk estimation. However for those who appeared to be unaffected there is no such 'gold standard'. Therefore, following a negative screen, the new clinical data were added in to a further computer calculation in order to reassign the risk estimation to produce a final, or residual, risk. All individuals diagnosed affected by the current round of screening were included in this calculation, thereby improving linkage phase information. The residual risk was calculated in some cases as less than that of the general population, thereby effectively excluding FAP and serving as a hard end-point. In others the residual risk was sufficiently low to influence screening policy for their future care.

Results

Table 3 shows the prior risk, probe derived risk, combined probe and prior risk and the residual risk for those screened negative on the current round of examinations. It has been possible to assign risk estimation derived from genotype data for the majority of the study population. One or more probes were informative in all but two cases. The frequency of probe informativity ranked in descending order was pi227(83%; 95% CI, 71.2-94.7%), YN5.48(51%; 95% CI, 35.6-66.8%), L5.62(46%; 95% CI, 30.8-61.9%), C11P11(41%; 95% CI, 26.1-56.9%), EF5.44(24%; 95% CI, 11.0-37.8%), ECB27(2%; 95% CI, 0-7.3%). Data from informative flanking probes were obtained for 28 of the 41 individuals studied (68%; 95% CI, 53.8-82.8%). The use of pi227 and YN5.48 in combination was particularly efficient since at least one was informative in 35 instances (85%) and both were informative in 21 (51%) out of all 41 individuals tested.

An example family showing segregation of the chromosome carrying the mutant *APC* gene (d) and haplotypes for all probes informative in that family is shown in Figure 1. Genotype data for a deceased spouse in the second generation who died of breast cancer were obtained by extracting DNA from archival paraffin block material. Half-filled symbols indicate family members whose disease state was not established prior to this study. All those with a shaded haplotype were shown to be affected on the current round of screening while those screened negative are shown with an unshaded haplotype. On the initial calculation, a risk of 0.88 was assigned to the individual who inherited the chromosome with a recombination event between C11P11 and EF5.44 (No. 20 in Table 3). This risk is calculated because EF5.44 predicts an affected status while C11P11 predicts an unaffected status. Therefore, there has been a recombination event which could have occurred at any point in the interval EF5.44-C11P11 and the computer analysis takes this, and the respective recombination fractions, into account when assigning a risk estimation. This case serves to stress the value of data from flanking markers because if only C11P11 had been informative, the risk for that patient would have been calculated as only 0.07.

Fourteen of the 15 patients screened and shown to be affected (93%) had been assigned probe derived risks of 0.93 or greater. Four of these individuals (No's 2,19,20,34 in Table 3) had previously been discharged from follow-up on the basis of negative colonic screening in their late twenty's and thirty's. All 4 individuals are actually affected and one already had carcinoma *in situ* in the resected colon. These patients may well have developed infiltrative colorectal cancer had they not been part of this study. The level of combined risk in these cases is lower than the probe derived risk alone because of the earlier negative screening. These cases emphasise the importance of DNA analysis in minimising inadequacies in clinical screening.

An initial combined risk of 0.095 was assigned for one individual (No. 29) which was at variance with clinical findings that indicate he is affected. However, in this patient only one probe was informative resulting in a level of risk which would not have influenced clinical management.

Eighteen individuals were screened negative on the current round of examinations. Fourteen of these (78%) had been assigned probe derived risk estimations of around 0.01 or less and 16 (89%) assigned a risk of <0.10. Combining probe information and clinical data based on the negative findings at this examination, residual risk

estimations of 0.001 or less were assigned to 16/18 (89%). Ten individuals (56%) had residual risks substantially less than 0.0001, which is the incidence rate of FAP in the general population.

Discussion

We have demonstrated the practical clinical value of risk analysis using linked DNA probes. The validity of probe derived risk estimation has been demonstrated and we have shown that genotype information from a simple blood test, when combined with independent clinical data, provides an estimation of residual risk for each patient which can be used to influence future screening policy. The use of linked DNA markers can avoid inappropriate discharge of gene carriers after sub-optimal or foreshortened screening protocols. In this study population, 4 individuals aged between 30 and 40 years would almost certainly have developed colorectal cancer had tracing, genotyping, clinical screening and subsequent surgery not been undertaken. This serves to emphasise the need for continued colorectal surveillance until middle age in patients assigned high risk estimations or those with indeterminate risk.

A clear distinction must be made between what is an acceptable level of risk to inform a patient of the likelihood that he/she is affected and what is an acceptable level of risk to allow a reduction in screening. While an initial combined risk of 0.7 would allow the clinician to advise a patient that it is probable he/she is affected, we believe that a probe derived risk of 0.01 or less is necessary before any radical change in screening policy is undertaken. The rationale for selecting this level of risk is that an at-risk individual screened negative for polyps and for CHRPE on first examination at the age of 15 who has a probe derived risk of 0.01 has a 3 in 10,000 chance of being affected. We suggest that at this level it is reasonable to advise only a single further colonic examination at the age of 30 years when the residual risk will be only 0.00001 if he/she is again screened negative, ten times less than the incidence of the disease in the general population. For individuals aged 15 with no polyps and negative CHRPE who have a probe derived risk of 0.1-0.01, it would also be reasonable to reduce colonic screening frequency to once every 2-3 years since their residual risk is only 0.004.

The locus order used here is based on an amalgamation of independent data from rare recombination events[6,12], published linkage data[1-6,12] and deletion analysis in colorectal cancers[13]. The recombination fractions between marker and *APC* are somewhat greater than might be suggested by summation of published data but it is important not to place a marker over-optimistically close to *APC* and give ill-informed reassurance to patients. Liability classes have been assigned based on the age-of-onset curves of Murday[11] since it is the risk of developing polyps after being screened negative which is of importance to risk analysis. These penetrance values are substantially different from those suggested by curves derived from age of *presentation* of polyps which are the data usually quoted[20].

Flanking markers were informative in 68% of individuals tested with a 95% confidence interval of 53.8-82.8%. Risk estimation is extremely accurate when flanking markers are informative and the risk assessments derived from each probe concur. In practice the phenomenon of genetic interference (where there is a selection against the occurrence of two recombinations close to each other) ensures an even greater diagnostic accuracy than that suggested by the computer calculated risk shown here. Recombinations between flanking markers were identified in four individuals (No's. 20, 30, 34, 39 in Table 3). The value of informative flanking markers cannot be over-stressed since recombination events can be recognised and taken into account in the risk calculation. If only a single marker is informative then, in a proportion of patients (dependent on recombination fraction), crossovers which occur between that marker and APC will not be recognised.

Individuals at-risk of FAP and their parents universally welcomed a simple blood test with the potential to give an estimation of the risk of having inherited the mutant gene. It was clear that prolonged and repeated examination of the colorectum was a burden to all those who required it. Indeed one of the study group (individual 8) agreed willingly to the blood test but refused to have any colonic or ophthalmological examination despite counselling. Fortunately, his genotype suggests he is unaffected although he and his children will be included in FAP screening programs with regular invitations for endoscopic follow-up.

One perinatal diagnosis (No. 40) was carried out at the parents request by genotyping DNA purified from placental tissue. Maternal contamination of the DNA could not influence this analysis since the mother was homozygous for flanking markers and she did not carry the marker allele which cosegregated with *APC* in her affected husbands family. The parents were counselled prior to the test and were fully aware of the implications of a test at such a young age. Unfortunately the child is almost certainly affected but the parents are glad to know this and felt they would have suffered from the uncertainty otherwise.

The data presented here demonstrate that linked DNA probe markers can now be used for preclinical risk estimations in FAP. Genotype data can be incorporated into current screening protocols to reduce the burden of colonic screening and to minimise the risk of gene carriers being lost to follow-up. We have been able to propose a policy for an integrated approach to screening individuals at risk of FAP, involving genotyping, retinal examination and colonic screening in suitable families. Linked DNA markers will continue to have a central role in presymptomatic diagnosis of FAP even after identification and cloning of the *APC* gene with subsequent characterisation of common mutations within it. Families which do not carry easily typed mutations will still require DNA probe derived risk estimations and linked markers will also be needed for confirmation of mutation analysis.

Acknowledgements

This work was partly funded by the Cancer Research Campaign. Computing hardware was funded by SHHD grant No. K/MRS/SO/C1387. We are indebted to the patients and physicians who contributed to this work, in particular to Professor CI Phillips for ophthalmoscopy examinations. Many thanks to Mr. S Gairns for fundal photography, Susan Holloway for advice on risk analysis and to those who kindly donated their probes and are referenced in the text.

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Table 1 Liability classes and age-dependent penetrance.

Liability class	Age (years)	Penetrance
1	0-9	0.0001
2	10-14	0.5
3	15-19	0.75
4	20-24	0.85
5	25-29	0.90
6	30-34	0.99
7	35+	0.999

Table 2 Allele size and frequency recognised by 6 DNA probes used in this study in 50 individuals from a local control population. The restriction endonuclease allowing recognition of the polymorphism at each probe locus is given in parentheses.

Probe (Enzyme recognising polymorphism)	Freq./size (kb)			Reference
	A1	A2	A3	
EF5.44(Msp1)	0.18/2.9	0.82/2.1		6
L5.62(BglII)	0.93/9.0	0.07/5.5		6
YN5.48(Msp1)	0.45/9.0	0.55/8.0		4,6
ECB27(BglII)	0.39/11.9	0.61/10.5		5,6
C11P11(Taq1)	0.14/4.4	0.86/3.9		1,2,6
pi227(Pst1)	0.25/3.0	0.75/4.3		3,6,14
pi227(BclI)	0.17/3.0	0.46/1.8	0.37/1.2	3,6,14
pi227(BstX1)	0.29/2.7	0.71/2.3		3,6,14
pi227(Mbo1)	0.25/0.55	0.75/0.45		3,6,14

Table 3 At-risk family member, prior risk and probe derived risk with DNA probes informative (P=pi227, C=C11P11, EC=ECB27, L=L5.62, EF=EF5.44, Y=YN5.48). Combined probe and prior risk is only indicated if prior risk was <0.5, otherwise combined risk is as for probe derived risk alone. The risk estimations are derived by computer analysis from single informative or closest flanking probes, screening modality (1=colonoscopy and biopsy, 2=CHRPE phenotype, 3=presence of gross osteomas) and final clinical diagnosis based on that method with residual risk in parentheses. ? indicates unknown clinical status (ie young child with unknown CHRPE status). The broken lines separate individuals from different families. The residual risk for individuals 27 and 28 was influenced when their father (26) was screened and shown to be affected.

At-risk subject	Prior risk	Any probe informative	Probes informative	Probe derived risk	Combined probe and prior risk (if prior risk <0.50)	Method of screening	Clinical status (Residual risk)
1	0.50	Y	P,C,Y	0.0040	-	1,3	-ve(<0.000007)
2	0.25	Y	P,C,EC,L,EF	0.9503	0.8636	1,2,3	affected
3	0.15	Y	P,Y	0.0032	0.00056	1,3	-ve(0.000003)
4	0.10	Y	P,Y	0.0032	0.00036	1,3	-ve(0.000003)
5	0.25	Y	P,L,Y	0.0006	0.0002	1,3	-ve(0.000006)
6	0.10	Y	P,Y	0.0032	0.00036	1,3	-ve(0.000036)
7	0.50	Y	P,Y	0.9968	-	1,2,3	affected
8	0.50	Y	Y	0.0865	-	(declined)	?(0.0865)
9	0.25	Y	P,C	0.0680	0.0237	1	-ve(0.0179)
10	0.25	Y	P,C	0.0680	0.0237	1	-ve(0.0179)
11	0.50	Y	P,L	0.0200	-	2	-ve(0.0011)
12	0.50	Y	P,L,EF	0.9998	-	2	affected
13	0.50	Y	P,L	0.9800	-	2	affected
14	0.50	Y	P,L,EF	0.9998	-	2	affected
15	0.50	Y	C,L,EF	0.0004	-	2	-ve(0.00002)
16	0.50	Y	C,L	0.0200	-	2	-ve(0.0011)
17	0.50	Y	C,L	0.0200	-	2	-ve(0.0011)
18	0.50	Y	C,L	0.9799	-	2	affected
19	0.10	Y	P,C,EF,Y	0.9991	0.9920	1,2	affected
20	0.15	Y	P,C,EF,Y	0.8777	0.5588	1,2	affected
21	0.25	Y	P,C,EF,Y	0.0007	0.0002	1,2	-ve(0.000017)
22	0.50	Y	P,C,Y	0.9798	-	1,2	affected
23	0.50	Y	P,Y	0.0026	-	1,2	-ve(0.00006)
24	0.50	Y	P,Y	0.9612	-	2	affected
25	0.50	Y	P,Y	0.9272	-	2	affected
26	0.50	N	-	-	-	1	affected
27	0.50	Y	P	0.4378	-	(not performed)	?(0.8757)
28	0.50	Y	P	0.1561	-	(not performed)	?(0.3122)
29	0.50	Y	P	0.0952	-	2	affected
30	0.50	Y	P,Y	0.7728	-	2	-ve(0.1518)
31	0.50	Y	P,Y	0.0032	-	2	-ve(0.0032)
32	0.50	Y	P,C,L,Y	0.00065	-	1,2	-ve(0.000018)
33	0.50	Y	P,C,L,Y	0.00065	-	1,2	-ve(0.000036)
34	0.25	Y	P,L	0.9128	0.7772	1,2	affected
35	0.50	Y	P,L,EF	0.9998	-	3	affected
36	0.50	Y	P,L,EF	0.9998	-	3	affected
37	0.50	Y	P,L,EF	0.9998	-	(not performed)	?(0.9998)
38	0.50	Y	P,C,L,Y	0.9994	-	(not performed)	?(0.9994)
39	0.50	Y	P,C,L,Y	0.5960	-	2	-ve(0.1408)
40	0.50	Y	P,C,L,Y	0.9994	-	(not performed)	?(0.9994)
41	0.50	N	-	-	-	(not performed)	?(0.5)